UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA

Departamento de Bioquímica y Biología Molecular II

CICLO CELULAR Y ENFERMEDAD DE ALZHEIMER: ESTUDIOS EN CÉLULAS EXTRANEURONALES DE PACIENTES Y EN MODELOS MURINOS

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR

Noemí Esteras Gallego

Bajo la dirección de la doctora

Ángeles Martín Requero

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Memoria para optar al grado de Doctor presentada por

NOEMÍ ESTERAS GALLEGO

Realizada en el Centro de Investigaciones Biológicas (CSIC) bajo la dirección de la Doctora Ángeles Martín Requero.

Madrid, 2012

VºBº DIRECTORA DE TESIS

Dra. Ángeles Martín Requero

VºBº INTERESADA

Noemí Esteras Gallego

MINISTERIO
DE ECONOMÍA
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CIENTÍFICAS

Dª. ÁNGELES MARTÍN REQUERO, Investigadora Científica en el Centro de Investigaciones Biológicas, del Consejo Superior de Investigaciones Científicas,

CERTIFICA

Que Dª NOEMÍ ESTERAS GALLEGO, licenciada en Farmacia por la Universidad Complutense de Madrid, ha realizado bajo mi dirección el proyecto correspondiente a su Tesis **"Ciclo celular y enfermedad de Alzheimer. Estudios en células extraneurales de pacientes y en modelos murinos"**, y que reúne los requisitos necesarios para su presentación como Tesis Doctoral en el Departamento de Bioquímica y Biología Molecular II de la Universidad Complutense de Madrid.

LA DIRECTORA,

Fdo:
Dª Ángeles Martín Requero

La presente tesis se presenta en Formato Publicaciones, como una colección de cinco artículos, cuatro de ellos ya publicados en revistas internacionales y el último enviado y pendiente de publicación.

El trabajo aquí presentado ha sido realizado con cargo a los proyectos de Investigación siguientes:

SAF2007-62405 Dirección General de Investigación Científica y Técnica. Plan Nacional (2007-2010). "*Interacciones entre Ca2+/Calmodulina y las rutas de supervivencia/muerte en células extraneurales de pacientes de Alzheimer y otras demencias"*

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JAEPRE2008 del Programa "Junta de Ampliación de Estudios" del CSIC.

A mi madre

A veces podemos pasarnos años sin vivir en absoluto, y, de pronto, toda nuestra vida se concentra en un solo instante.

Oscar Wilde

Descubrí el secreto del mar meditando sobre una gota de rocío.

Antonio Machado

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Agradecimientos

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Abreviaturas

Abreviaturas

Aβ - β-amiloide

Ach - Acetilcolina

AchEI - Inhibidores de acetilcolinesterasa *(AcetylcholinEsterase Inhibitors)*

ADC - Coeficiente de difusión aparente *(Apparent Diffussion Coeficient)*

ADN - Ácido desoxirribonucleico

AICD - Dominio Citoplasmático de la Proteína Precursora de Amiloide *(Amyloid Precursor Protein Cytoplasmic Domain)*

AINEs - AntiInflamatorios No Esteroideos

APP - Proteína precursora de amiloide *(Amyloid Precursor Protein)*

ATP - Adenosín trifosfato

ARN - Ácido ribonucleico

BACE 1 - Enzima de procesamiento de APP en el sitio β, β-secretasa *(Beta-site APP Cleaving Enzyme 1)*

BDNF - Factor neurotrófico derivado del cerebro (*Brain-Derived Neurotrophic Factor)*

BrdU - 5'-bromo-2'-deoxiuridina

^{11}C-PiB - Compuesto B de Pittsburgh unido a carbono 11 *(^{11}C-labelled Pittsburgh compound B)*

CaM - Calmodulina

CaMBP - Proteína de unión a CaM (*CaM-Binding Protein)*

CaMKII - Ca $^{2+}$/Calmodulina proteína quinasa II (*Calcium/calmodulin dependent protein Kinase II)*

CAT - ColinAcetilTransferasa

Cdk - Quinasa dependiente de ciclina *(Cyclin-dependent Kinase)*

Cdk5rap1 - *(CDK5 Regulatory subunit-Associated Protein 1)*

Cdkn1a - Inhibidor de Cdk 1a o p21 *(CDK inhibitor 1a)*

Cdkn2a - Inhibidor de Cdk 2a o p16 *(CDK inhibitor 2a)*

Cip/Kip - Inhibidor de Cdk (*Cdk Interacting Protein/Kinase Inhibitory Protein)*

Cho - Colina *(Choline)*

Abreviaturas

CMSP - Células Mononucleares de Sangre Periférica

Cr - Creatina

CTF - Fragmento C terminal *(C-terminal Fragment)*

DCL - Deterioro Cognitivo Leve

DCX - Doblecortina *(Doublecortin)*

DISC - Complejo de señalización inductor de muerte *(Death-Inducing Signaling Complex)*

EA - Enfermedad de Alzheimer

EMA - Agencia Europea del Medicamento *(European Medicines Agency)*

ERK - Quinasa regulada por señales extracelulares *(Extracellular-signal Regulated Kinase)*

FBS - Suero fetal bovino *(Fetal Bovine Serum)*

FDA - Administración de Medicamentos y Alimentos de EEUU *(Food and Drug Administration)*

FOXO3a - Factor de transcripción de la familia *forkhead* (*Forkhead Box O 3a)*

FPP - Farnesilpirofosfato

GFAP - Proteína Fibrilar Acídica Glial *(Glial Fibrillary Acidic Protein)*

GGPP - Geranilgeranilpirofosfato

Gpr132 - Receptor 132 acoplado a proteína G *(G Protein-coupled Receptor 132)*

GSK3β - Glucógeno sintasa quinasa β

HMG CoA - 3-hidroxi-3-metilglutaril-Coenzima A

INK4 - Inhibidores de la quinasa dependiente de ciclina 4 *(Inhibitors of Cyclin D-dependent Kinase 4)*

iPSC - Célula madre con pluripotencialidad inducida *(Induced Pluripotent Stem Cell)*

MAPK - Proteína quinasa activada por mitógeno *(Mitogen-Activated Protein Kinase)*

MDM2 - *Murine-Double Minute 2*

mI - Mioinositol

MMSE - Mini examen del estado mental *(Mini-Mental State Examination)*

Abreviaturas

MRI - Imagen de Resonancia Magnética *(Magnetic Resonance Imaging)*

MRS - Espectroscopía de Resonancia Magnética *(Magnetic Resonance Spectroscopy)*

MT - Transferencia de magnetización *(Magnetization Transfer)*

NAA - N-Acetilaspartato

Nek2 - *NIMA (Never In Mitosis gene A)-related Expressed Kinase 2*

NGF - Factor de crecimiento nervioso *(Nerve Growth Factor)*

NINCDS-ADRDA - Instituto Nacional para los Desórdenes Neurológicos, Comunicativos y Accidente Cerebro Vascular - Asociación para la Enfermedad de Alzheimer y Desórdenes Asociados *(National Institute of Neurologic, Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association)*

NMDA - N-Metil D-Aspartato

PCNA - Antígeno nuclear de proliferación celular (*Proliferating Cell Nuclear Antigen)*

PDGF - Factor de crecimiento derivado de plaquetas *(Platelet-Derived Growth Factor)*

PET - Tomografía por emisión de positrones *(Positron Emission Tomography)*

PHF - Filamentos Helicoidales Pareados *(Paired Helical Filaments)*

PI3K - Fosfatidilinositol 3-quinasa *(Phosphatidylinositol 3-kinase)*

PPAR - Receptor Activado por Proliferadores de Peroxisomas (*Peroxisome Proliferator-Activated Receptor)*

PS, PSEN - Presenilina

qRT-PCR - Reacción en cadena de la polimerasa cuantitativa en tiempo real *(Quantitative Real Time Polymerase Chain Reaction)*

ROS - Especies Reactivas de Oxígeno *(Reactive Oxygen Species)*

SERCA - Calcio ATPasa del retículo sarco/endoplásmico *(Sarco/Endoplasmic Reticulum Calcium ATPase)*

SNC - Sistema Nervioso Central

TGF-β - Factor de crecimiento transformante β *(Transforming Growth Factor* β*)*

TNF - Factor de necrosis tumoral *(Tumor Necrosis Factor)*

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Introducción

1

1 Breve descripción de la enfermedad de Alzheimer

La demencia es un síndrome clínico que se caracteriza por un deterioro progresivo de las funciones cerebrales superiores como son la memoria, el lenguaje, la orientación, el cálculo o la percepción espacial y que conlleva una pérdida de autonomía del enfermo, que se vuelve cada vez más dependiente a medida que avanza la enfermedad.

Aunque la demencia era conocida desde la antigüedad, no fue hasta 1906 cuando el médico alemán Alois Alzheimer sentó las bases biológicas de la enfermedad que ahora lleva su nombre y que constituye la principal causa de demencia a nivel mundial. Su paciente, Auguste D., de 51 años, presentaba un cuadro clínico caracterizado por pérdida progresiva de memoria, desorientación en el tiempo y el espacio, paranoia y trastornos en la conducta y el lenguaje. Tras su muerte, Alzheimer examinó su cerebro mediante tinción de plata y describió lo que a día de hoy suponen las dos principales características neuropatológicas de la enfermedad: "depósitos de una sustancia peculiar en el córtex" (ahora conocidos como placas neuríticas o seniles) y "cambios peculiares en las neurofibras" (denominados en la actualidad ovillos neurofibrilares) (figura 1).

Más de un siglo después, cuando la esperanza de vida al nacer ha aumentado de manera considerable, la enfermedad de Alzheimer (EA) se ha convertido en la principal causa de demencia en todo el mundo en personas mayores de 65 años.

1 Breve descripción de la enfermedad de Alzheimer

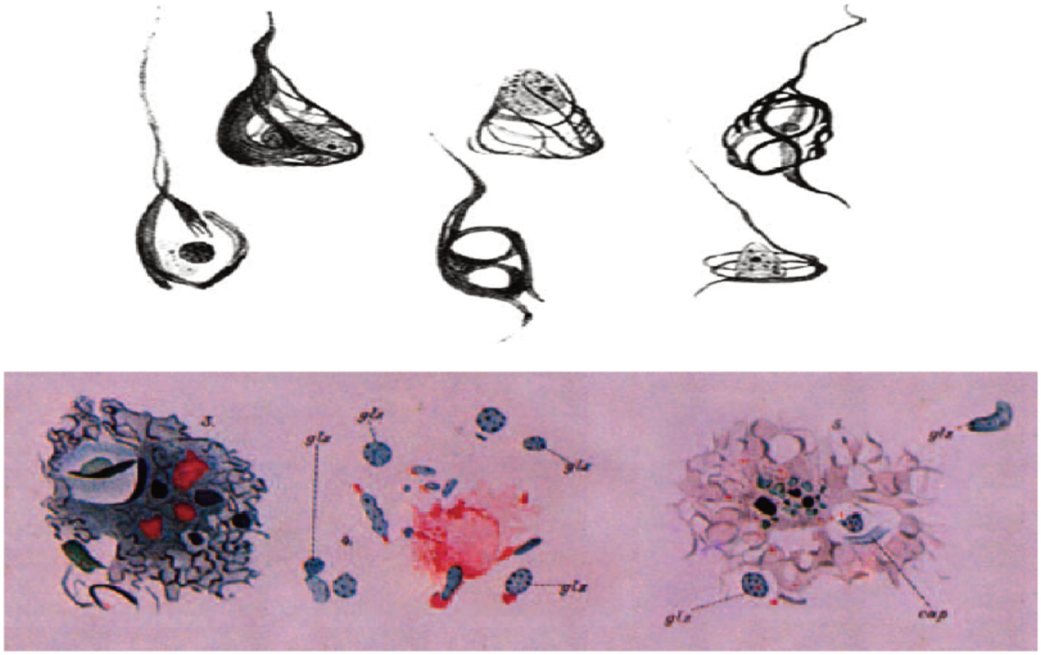

Figura 1. Dibujos originales de Alois Alzheimer. En primer lugar están representados los ovillos neurofibrilares y debajo las placas neuríticas.

Aunque los datos son difíciles de cuantificar, debido a la falta de estudios rigurosos en algunas regiones, el estudio Delphi (Ferri y cols., 2005) estimaba que en 2001 el número de personas con demencia en todo el mundo llegaba a los 24 millones, con una previsión de que ese número se doble cada 20 años, llegando a alcanzar los 80 millones de personas en 2040. Aunque en la actualidad la mayoría de los casos de demencia se describen en países desarrollados, se estima que en unos años el porcentaje de enfermos en países en desarrollo será 4 veces mayor que en países desarrollados.

Además, la EA, por sus características, constituye un problema familiar, social y económico de primera magnitud. A medida que progresa la enfermedad, el enfermo se vuelve cada vez más dependiente y necesita de mayores cuidados. En la mayoría de los casos, sobre todo en la sociedad española y en las latinas en general, los responsables del cuidado del paciente son los familiares. A medida que la enfermedad progresa en el paciente, la presión sobre el cuidador se incrementa. Debido a esta situación, los cuidadores también muestran un aumento en la frecuencia de enfermedades psicológicas y físicas (ansiedad, depresión, aislamiento social, síntomas somáticos) y una importante reducción de su calidad de vida, ligada invariablemente a la del paciente.

En cuanto a los costes económicos asociados a la EA, se pueden distinguir dos tipos: di-

rectos e indirectos. Los directos son gastos cuantificables que derivan del cuidado del paciente y que engloban el gasto sanitario, los fármacos, la atención médica, la institucionalización, etc., y suponen alrededor del 20% del coste total. Por otro lado, los gastos indirectos o sociales, son difícilmente cuantificables (tiempo dedicado al cuidado del paciente por parte del entorno, pérdida de productividad de sus cuidadores, etc.) y representan alrededor del 80% del total. Estos gastos indirectos son asumidos principalmente por las familias. En las fases finales, se produce un aumento en los costes directos que responde principalmente a la institucionalización del paciente. Un estudio reciente (Coduras y cols., 2010) calcula que el coste medio derivado del cuidado de una persona que sufre EA supone en España alrededor de 1400 euros mensuales, asumidos en un 88% por la propia familia. El 12% restante corresponde a gastos directos que son asumidos por los fondos públicos.

2 Patogénesis de la enfermedad

Uno de los aspectos que merece la pena resaltar sobre la descripción que hizo Alzheimer de la enfermedad en 1906, fue el hecho de que realizase un estudio neuropatológico del cerebro y que propusiera que el comportamiento de la paciente era consecuencia de un depósito anormal en su cerebro, convirtiéndose así en un pionero en ligar estructura del cerebro y función. En la actualidad se reconocen dos principales características neuropatológicas de la EA: las placas neuríticas y los ovillos neurofibrilares. Ambas han sido consideradas hasta ahora el "estándar de oro", es decir, solamente la confirmación post-mortem de su presencia en el cerebro del paciente, permite hacer un diagnóstico definitivo de la enfermedad. Otras características bien definidas son la muerte neuronal y las alteraciones sinápticas.

2.1 Placas neuríticas

Las placas neuríticas o seniles son depósitos proteicos extracelulares, de morfología más o menos esférica y con un tamaño de entre 10 y 200 µm de diámetro (figura 2). Durante la década de los ochenta tuvieron lugar dos importantes avances en el estudio de la EA. En primer lugar, la secuenciación del principal componente de las placas, un péptido de 4,2 KDa, formado por entre 40 y 42 aminoácidos: el péptido β-amiloide (Aβ, *Amyloid β)* (Glenner y Wong, 1984). Se postulaba que este péptido provenía del procesamiento de un

2 Patogénesis de la enfermedad

precursor de mayor tamaño, y en efecto, tres años más tarde, se clonó un receptor de superficie cuyo procesamiento aberrante parecía ser el responsable de los depósitos de amiloide en el cerebro: la proteína precursora de amiloide (APP, *Amyloid Precursor Protein*) (Kang y cols., 1987) .

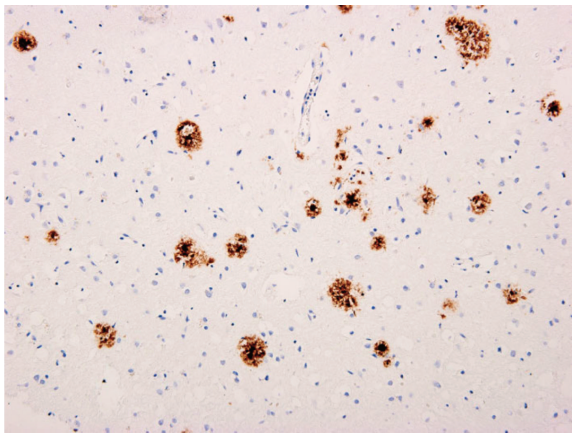

Figura 2. Placas neuríticas en el cerebro de un paciente de EA. La figura muestra la presencia de las placas en la corteza temporal de un paciente de enfermedad de Alzheimer. Inmunohistoquímica contra β-amiloide (4G8). Tomado de (Perl, 2010).

El gen *APP* se localiza en el cromosoma 21 y da lugar a numerosas isoformas por *splicing* alternativo y modificaciones post-transcripcionales, siendo las más comunes la de 695 aminoácidos (que se expresa principalmente en el sistema nervioso) y las de 751 y 770, que se expresan de forma ubicua (Bayer y cols., 1999). La secuencia de *APP* ha sido altamente conservada durante la evolución en mamíferos, y la proteína pertenece a una familia que incluye APLP1 y APLP2 (*Amyloid Precursor-Like Proteins*) en mamíferos y APPL en Drosophila (*Amyloid Precursor Protein-Like*) (O'Brien y Wong, 2011). Todas ellas son proteínas transmembrana con amplios dominios extracelulares y se procesan del mismo modo, pero sin embargo, sólo APP genera el fragmento amiloidogénico.

2.1.1 Procesamiento de APP

Existen diferentes rutas para el procesamiento de APP, que pueden dar lugar o no a la formación del péptido Aβ, como se puede observar en la figura 3.

La **vía no amiloidogénica**, la que no genera Aβ, comienza con la acción de una α-secretasa, que realiza un corte en el dominio extracelular, a nivel del aminoácido 687, liberando el extremo extracelular soluble, sAPPα. A continuación, el fragmento C-terminal α restante (CTF-α, *Carboxyl-Terminal Fragment* α) de 83 aminoácidos es procesado por una γ-secretasa que libera un pequeño fragmento extracelular (P3) y otro intracelular (AICD, *Amyloid Precursor Protein Cytoplasmic/C-terminal Domain*).

Por su parte, la **vía amiloidogénica**, comienza con la acción de una β-secretasa, que realiza el primer corte a nivel del aminoácido 671, generando un fragmento extracelular soluble, sAPPβ y un fragmento C-terminal de 99 aminoácidos. A continuación, el fragmento CTF-β es procesado por la γ-secretasa dando lugar a un fragmento extracelular, el β-amiloide, y a un fragmento C-terminal intracelular, AICD, idéntico al

generado por la vía no-amiloidogénica. La γ-secretasa corta APP en múltiples sitios en un proceso secuencial para generar péptidos de Aβ de diferente longitud. La mayoría son de 40 ó 42 aminoácidos, pero el tamaño puede variar entre 38 y 43. El péptido de 40 aminoácidos es el más común, pero el de 42 es el más hidrofóbico y probablemente el que favorece la oligomerización, la formación de fibras y finalmente de placas (Iwatsubo y cols., 1994).

Las secretasas que forman parte de estas rutas de procesamiento han sido objeto de una exhaustiva caracterización. Actualmente se atribuye

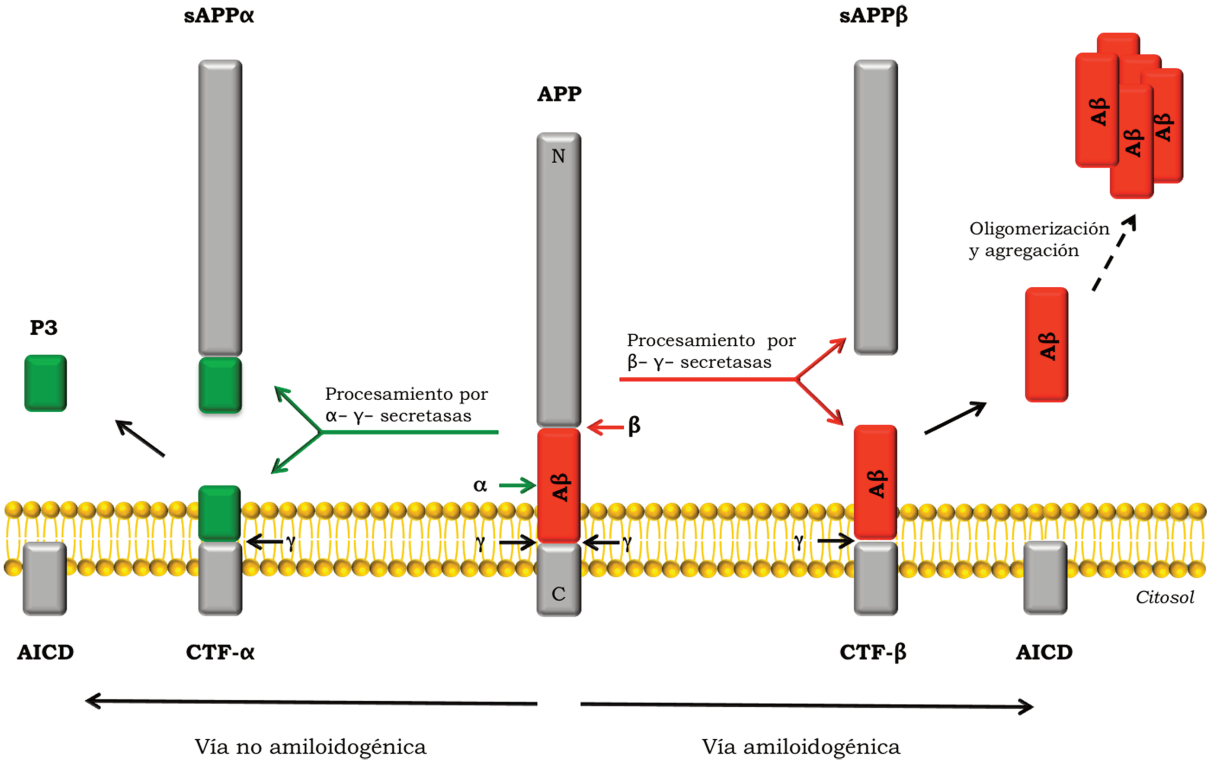

Figura 3. Esquema del procesamiento proteolitico de APP. El procesamiento a través de la β y la γ secretasas genera el fragmento Aβ amiloidogénico, mientras que la α y la γ secretasas dan lugar a una vía no amiloidogénica. Modificado de (Cole y Vassar, 2006).

la actividad α-secretasa a la familia de metaloproteasas ADAM (*A Disintegrin And Metalloproteinase*), siendo ADAM10 la que parece regular el proceso (Hooper y Turner, 2002). El procesamiento por la α-secretasa es además de un gran interés desde el punto de vista terapéutico. La activación preferente de esta ruta evitaría la producción de Aβ, ya que el corte por α-secretasa se produce dentro de su secuencia.

La actividad β-secretasa, por su parte, es llevada a cabo por la proteasa transmembrana BACE1 (*Beta-site APP Cleaving Enzyme 1*) (Cai y

2 Patogénesis de la enfermedad

cols., 2001). Se considera que la acción de BACE1 es el paso limitante para la producción de Aβ, con lo que el estudio de posibles inhibidores de su función parece prometedor como estrategia terapéutica. Sin embargo, entre otras dificultades de tipo estérico, se ha demostrado que BACE1 tiene multitud de sustratos fisiológicos, de modo que la inhibición de esta enzima conlleva importantes efectos colaterales que están dificultando los estudios (Klaver y cols., 2010).

La γ-secretasa es un amplio complejo proteasa formado por varias proteínas de membrana distintas: presenilina 1 (PS1), presenilina 2 (PS2), nicastrina, Aph-1 y Pen2, siendo las presenilinas las que poseen la actividad catalítica (De Strooper y cols., 2012). Además, este complejo es esencial para la proteólisis de otros muchos sustratos, entre ellos Notch, que tras el procesamiento por la γ-secretasa libera su dominio intracelular, NICD *(Notch Intracellular Domain)*, una molécula que puede transactivar muchos genes críticos para el desarrollo (Schroeter y cols., 1998). Por este motivo, los ratones knock-out para PS1 no son viables (Shen y cols., 1997).

2.1.2 Funciones de APP

La función fisiológica de APP aún no se conoce con exactitud pero numerosos estudios han demostrado que el producto fisiológico del procesamiento de APP, el fragmento soluble sAPPα, está implicado en neuroprotección, plasticidad sináptica, crecimiento de las neuritas y sinaptogénesis (Kogel y cols., 2011). A pesar de ello, la deleción de *APP* en ratones apenas tiene consecuencias vitales, por lo tanto, no parece haber evidencia de que se produzca una pérdida de función celular de APP en pacientes de alzhéimer, sino que la proteína intervendría en la enfermedad por el incremento en la producción del fragmento Aβ citotóxico (Selkoe, 1998).

2.1.3 Formación de las placas de amiloide.

Aβ se secreta de manera constitutiva en células normales en cultivo y se detecta como péptido circulante en plasma y en el líquido cefalorraquídeo (Haass y cols., 1992; Seubert y cols., 1992). El modelo propuesto para la formación de las placas de amiloide establece que, en condiciones fisiológicas, la neurona produce y secreta Aβ en forma de monómeros y trímeros, que una vez en el fluido intersticial se expanden de forma controlada. Sin embargo, cuando por alguna razón el monómero se despliega, permite al péptido formar dímeros que darán lugar a las protofibrillas y a las placas de amiloide (Larson y Lesne, 2012).

2.1.4 Tipos de placas de amiloide

❍ *Depósitos difusos de Aβ*

Este tipo de depósitos son poco reactivos, de tamaño grande (entre 50-500 μm) y con una es-

tructura poco organizada (Duyckaerts y cols., 2009). Además de en pacientes, este tipo de placas se han encontrado en autopsias de personas que no tenían signos de demencia, a partir de los 40-50 años (Arai y cols., 1999). En algunos casos, estos depósitos en la mediana edad podrían representar una etapa preclínica de EA, pero no en todos ellos, ya que también se han encontrado placas difusas en muchas personas de edad avanzada sin signos de demencia (Bennett y cols., 2006). Algunos estudios indican que en personas sanas, el depósito de Aβ aumenta con la edad y que la frecuencia del alelo Apoε4 es mayor que en personas sin los depósitos (Rodrigue y cols., 2012).

❍ *Depósitos focales*

Además de los depósitos difusos, estudios inmunohistoquímicos muestran acúmulos densos y esféricos de Aβ, denominados depósitos focales, que están formados mayoritariamente por el péptido Aβ42 (Guntert y cols., 2006). En estos casos, los depósitos están casi siempre acompañados de células de microglía activadas (Arends y cols., 2000). Los depósitos focales pueden estar rodeados de una corona neurítica, rodeada a su vez por procesos astrocíticos, constituyendo así las placas maduras, clásicas o neuríticas. El componente neurítico de la corona está formado por neuritas distróficas, principalmente axones, que contienen grandes cantidades de lipofuscina, cuerpos densos, mitocondrias, filamentos helicoidales pareados formados por tau y en algunos casos ubiquitina (Duyckaerts y cols., 2009).

Se cree que los depósitos comienzan siendo difusos, después focales, y finalmente se rodean de la corona neurítica para formar las placas neuríticas (Metsaars y cols., 2003).

También hay que destacar que los depósitos de Aβ no se producen al azar, sino que se distribuyen de manera jerárquica y secuencial en el cerebro. Se ha descrito que comienzan en el neocórtex y luego se expanden a otras regiones en distintas fases (hipocampo, córtex cingulado y entorrinal, amígdala, tálamo e hipotálamo, ganglios basales y finalmente cerebelo) (Thal y cols., 2008a).

❍ *Depósitos vasculares*

En más del 80% de los pacientes de EA, y también en personas mayores sanas, la acumulación de Aβ también tiene lugar en las paredes de los vasos cerebrales, principalmente arterias, dando lugar a lo que se conoce como angiopatía amiloide cerebral (Thal y cols., 2008b). En casos severos, este tipo depósitos pueden estar asociados con hemorragias intracerebrales

2.2 Ovillos neurofibrilares

Los ovillos neurofibrilares, también descritos por Alzheimer en 1906, son agregados pro-

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teicos presentes en el interior de las neuronas, concretamente en el pericarion (citoplasma de la neurona) y en las dendritas proximales. En 1963, la microscopía electrónica permitió observar que los ovillos estaban formados por unas estructuras denominadas filamentos helicoidales pareados (Kidd, 1963). Pero no fue hasta 1986 cuando se describió por primera vez su principal componente: la proteína asociada a los microtúbulos tau, (figura 4) (Grundke-Iqbal y cols., 1986).

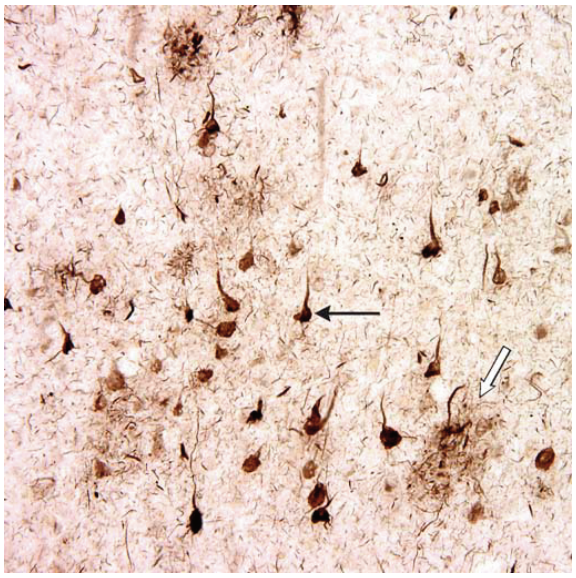

Figura 4. Ovillos neurofibrilares en un paciente de enfermedad de Alzheimer. La inmunohistoquímica muestra los ovillos neurofibrilares (flecha negra) y las placas neuríticas (flecha blanca). Tomado de (Binder y cols., 2005).

Tau es una molécula altamente soluble presente en todas las células nucleadas y su principal función es la estabilización de los microtúbulos, principalmente en los axones. Tau tiene abundantes sitios potenciales de fosforilación, y el balance fosforilación/defosforilación regula sus funciones fisiológicas (Obulesu y cols., 2011). Las quinasas que pueden fosforilar *in vivo* esta proteína se dividen en dos grupos; por una parte las quinasas que fosforilan residuos serina o treonina seguidos de prolina (Ser-Pro o Thr-Pro) y por otro lado las que fosforilan serina o treonina no seguidas de prolina. Dentro del primer grupo encontramos la glucógeno sintasa quinasa 3β (GSK3β) y la quinasa dependiente de ciclina 5 (Cdk5); y dentro del segundo la proteína quinasa A y la B (PKA y Akt respectivamente), la quinasa dependiente de calcio/calmodulina II (CaMKII, *Calcium/calmodulin dependent protein Kinase II*) o la quinasa reguladora de afinidad de microtúbulos (MARK) (Johnson y Stoothoff, 2004; Mi y Johnson, 2006; Dolan y Johnson, 2010). Por otro lado, la principal fosfatasa encargada de la defosforilación de tau es la proteína fosfatasa 2A (PP2A).

Cuando tau es anormalmente hiperfosforilada, principalmente en los residuos Ser-Pro o Thr-Pro, pierde su actividad biológica, se disocia de los microtúbulos, se hace resistente a la degradación, se inducen cambios conformacionales que la hacen insoluble y finalmente polimeriza hasta formar los filamentos helicoidales pareados (PHF, *Paired Helical Filaments)*, que son el principal componente de los ovillos neurofibrilares (Meraz-Rios y cols., 2010; Metcalfe y Figueiredo-Pereira, 2010). Además de la hiperfosforilación, el procesamiento de tau por caspasas o calpaína es otra modificación que juega un papel importante en la forma-

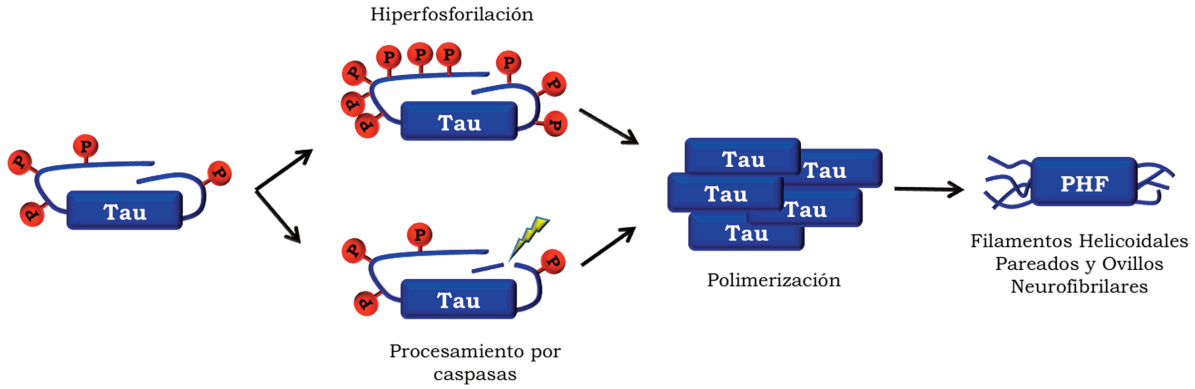

Figura 5. Oligomerización de tau y formación de ovillos neurofibrilares. Aunque en condiciones fisiológicas tau está fosforilada, la hiperfosforilación o su truncamiento tras el procesamiento por caspasas, facilitan la agregación y aumentan su toxicidad. Los monómeros patológicos forman oligómeros, que finalmente forman fibras, filamentos helicoidales pareados y ovillos neurofibrilares. Modificado de (Pritchard y cols., 2011).

ción de los PHF, ya que el truncamiento de la proteína favorece la formación de filamentos, ver figura 5 (Gamblin y cols., 2003).

La distribución de los ovillos sigue un patrón bien definido, que permitió establecer lo que se conoce como estadíos de Braak, que además se correlacionan con la evolución clínica de la enfermedad. Los estadíos I y II son etapas preclínicas, aún no existe deterioro cognitivo y los ovillos aparecen en la corteza entorrinal, la transentorrinal y el hipocampo. En los estadíos III y IV los ovillos aparecen también en el sistema límbico y la enfermedad se ha hecho patente. Por último, los estadíos V y VI se correlacionan con la fase más severa de la enfermedad, y los ovillos se observan también en el neocórtex (Braak y Braak, 1991).

La proteína tau no solamente se acumula en la EA. También se observan acúmulos de tau en un grupo heterogéneo de demencias denominadas taupatías, en las que por el contrario no aparecen placas de amiloide. Ejemplos de taupatías son la parálisis supranuclear progresiva , la demencia frontotemporal con parkinsonismo asociada al cromosoma 17, la degeneración corticobasal o la enfermedad de Pick (Robert y Mathuranath, 2007).

2.3 Muerte neuronal

Otra característica principal de la enfermedad es la muerte selectiva de poblaciones neuronales en el cerebro. Esta alteración neuropatológica se caracteriza por la vulnerabilidad de determinadas neuronas en áreas concretas del cerebro. En la EA, el neocórtex y el hipocampo son las zonas principalmente afectadas, siendo las neuronas piramidales de la corteza entorrinal y de la región CA1 y el subículo del hipocampo las más vulnerables a la neurodegeneración (revisado en

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(Morrison y Hof, 1997). La corteza entorrinal recibe información altamente procesada de las áreas de asociación de la corteza (relacionadas con las funciones cognitivas y afectivas) y la transmite al giro dentado del hipocampo a través de la denominada vía perforante, que juega así un papel muy importante en la memoria. La capa II de la corteza entorrinal (de donde parte la vía perforante) sufre una extensa muerte neuronal ya en estadios iniciales de la EA, que puede llegar al 90% en EA avanzada (Gomez-Isla y cols., 1996; Price y cols., 2001). De esta manera, la disrupción de la vía perforante puede explicar algunos aspectos de la alteración de la memoria en la EA (Hyman y cols., 1986). Los tipos neuronales afectados en la EA son especialmente sensibles a la deprivación energética, lo que unido al resto de estímulos estresantes que rodean la enfermedad, puede ser una de las causas de la vulnerabilidad selectiva de estas células (Saxena y Caroni, 2011). La muerte neuronal, junto con los ovillos neurofibrilares, es una característica fisiopatológica que se correlaciona muy bien con el déficit cognitivo (Giannakopoulos y cols., 2003; Donev y cols., 2009).

2.3.1 Mecanismos moleculares de muerte neuronal

Los tres principales mecanismos de muerte neuronal son la necrosis, la apoptosis y la autofagia. Mientras que la necrosis es un tipo de muerte aguda y no programada, caracterizada por la lisis celular y la inflamación causada por la liberación de los componentes celulares al medio extracelular, la apoptosis y la autofagia son mecanismos controlados y programados de muerte celular.

2.3.1.1 Apoptosis

Numerosas investigaciones apuntan a la apoptosis como el principal mecanismo de muerte neuronal en la EA. Morfológicamente, la apoptosis se caracteriza por la condensación de la cromatina, la disminución del tamaño nuclear, la compactación del citoplasma y la fragmentación del ADN que da lugar a los cuerpos apoptóticos, fragmentos nucleares rodeados de membrana que son finalmente fagocitados sin signos de lisis ni inflamación (Saraste y Pulkki, 2000).

Bioquímicamente, la apoptosis está asociada a la activación de las caspasas, una familia de proteasas capaces de procesar una gran variedad de sustratos para finalmente dar lugar a la muerte celular (Fischer y cols., 2003). La familia de las caspasas está formada en mamíferos por 13 miembros, de los cuales algunos tienen funciones relacionadas con la apoptosis, mientras que otras son caspasas pro-inflamatorias (Martinon y Tschopp, 2007). Dentro de las que tienen función apoptótica, se pueden clasificar en caspasas iniciadoras (caspasa-8, -9 y -10) o efectoras (caspasas -3, -6 y -7) (Pop y Salvesen, 2009). La activación de las caspasas está altamente regulada, por lo que se sintetizan como precursores en forma de pro-caspasas que necesitan ser activadas. Las caspasas iniciadoras se activan por dimerización (Bo-

atright y cols., 2003) y activan a su vez a las caspasas efectoras por proteólisis (Boatright y Salvesen, 2003).

El proceso apoptótico se puede iniciar por dos vías bien definidas, la extrínseca y la intrínseca. La vía extrínseca está asociada a señales del medio extracelular recibidas a través de los receptores de muerte, mientras que la vía intrínseca actúa en respuesta a estímulos como daño en el ADN, radiación UV o ausencia de suero (Wang y Youle, 2009) y se inicia cuando se producen cambios en la permeabilidad de la membrana mitocondrial. Ésta se regula por la familia de proteínas Bcl-2, que incluye proteínas pro-apoptóticas (como Bax o Bak) o anti-apoptóticas (como Bcl-2 o Bcl-x_L) que promueven o inhiben la liberación de factores pro-apoptóticos al citosol (Shimizu y cols., 1999; Martinou y Youle, 2011). En la figura 6 se puede encontrar un resumen detallado del proceso apoptótico.

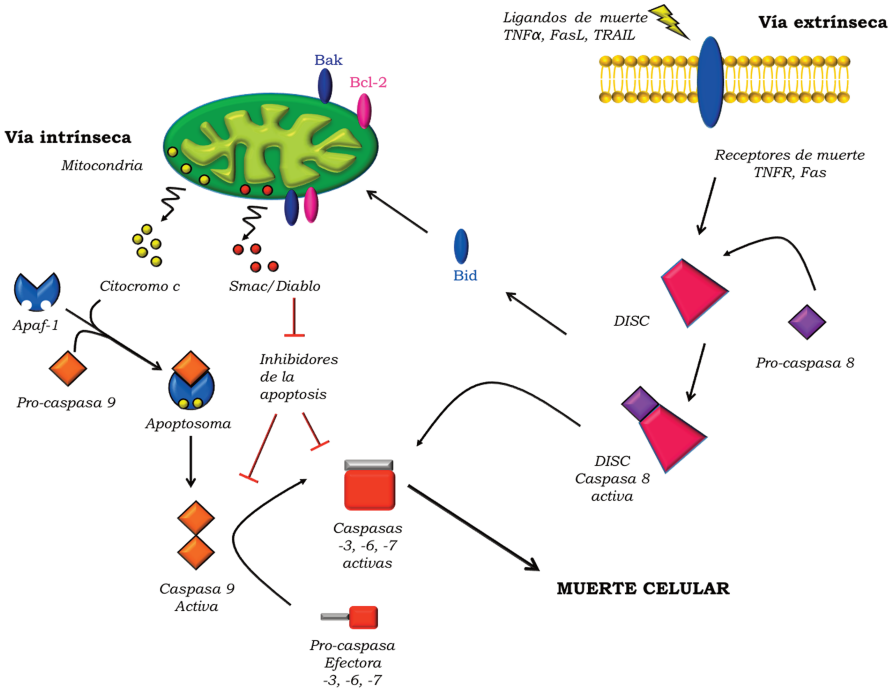

Figura 6. Vías intrínseca y extrínseca de activación de caspasas. La vía extrínseca (derecha) comienza con la unión de un ligando como FasL o TNF (*Tumor Necrosis Factor*) a un receptor de muerte, induciendo la formación del complejo DISC (*Death-Inducing Signalling Complex*), que recluta y activa la caspasa 8 iniciadora (Gonzalvez y Ashkenazi, 2010). La vía intrínseca (izquierda) comienza con la permeabilización de la mitocondria y la liberación al citosol de citocromo c, que permite el ensamblaje de Apaf1 (*Apoptosis Protease-Activating Factor*) y procaspasa 9, dando lugar al complejo activador de caspasas denominado apoptosoma y activando la caspasa 9 iniciadora (Li y cols., 1997; Gogvadze y cols., 2006). Junto con citocromo c, la mitocondria también libera las proteínas proapoptóticas Smac/Diablo, que actúan regulando negativamente la acción de un conjunto de proteínas inhibidoras de la apoptosis (Ekert y Vaux, 2005). La integridad de la membrana mitocondrial está regulada por la familia de proteínas Bcl-2, pero el complejo DISC de la vía extrínseca también puede potenciar la intrínseca permeabilizando la mitocondria a través de la proteína Bid. Ambas vías finalizan con la activación proteolítica por parte de las caspasas 8 y 9 de las caspasas efectoras 3, 6 y 7. Basado en (Ribe y cols., 2008).

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Numerosos hallazgos apoyan el papel de la apoptosis en la muerte neuronal en la EA, entre ellos el incremento de caspasas activas en el cerebro de pacientes, principalmente caspasa 3 (Arai y cols., 1999; Stadelmann y cols., 1999; Su y cols., 2001; Pompl y cols., 2003), caspasa 6 (Guo y cols., 2004; Graham y cols., 2011), caspasa 8 (Rohn y cols., 2001; Pompl y cols., 2003) y caspasa 9 (Rohn y cols., 2002; Pompl y cols., 2003).

Además, las caspasas también están implicadas en el procesamiento de tau. La hidrólisis de la proteína por caspasas da lugar a una forma truncada capaz de formar agregados y ovillos neurofibrilares más rápidamente que la proteína completa (Gamblin y cols., 2003; Rissman y cols., 2004). También APP puede ser sustrato de las caspasas y contribuir a la formación de Aβ (Gervais y cols., 1999), y en sentido contrario, el péptido Aβ es capaz de activar caspasas (Li y cols., 1996).

Algunos autores han planteado que el proceso de muerte neuronal en alzhéimer no se corresponde con el proceso apoptótico tradicional. La hipótesis se basa en que las células en cultivo emplean unas 24 horas en completar el proceso, algo que contrasta con una enfermedad crónica como el alzhéimer, lo que lleva a encontrar en un momento dado pocas neuronas que presenten signos morfológicos de apoptosis (Jellinger, 2006). Además, el hecho de que en las neuronas no se encuentren eventos terminales del proceso como condensación de cromatina o cuerpos apoptóticos, pero sí aparezcan signos tempranos de apoptosis como caspasas iniciadoras o fragmentación de ADN (Nunomura y Chiba, 2000), podría sugerir que una vez iniciada, la apoptosis no se completa, sino que se evita o al menos retrasa, un proceso que Raina y cols. denominaron abortosis (Raina y cols., 2001; Raina y cols., 2003). Este sería un mecanismo de supervivencia de las neuronas ante el ambiente pro-apoptótico en el que se encuentran, favorecido por la elevada presencia de proteínas anti-apoptóticas de la familia Bcl-2 en las neuronas de EA (Zhu y cols., 2004c; Rohn y Head, 2008).

2.3.1.2 Autofagia

El proceso de autofagia es un mecanismo por el cual algunas proteínas, agregados proteicos y orgánulos son degradados por la célula en los lisosomas. Los objetivos son dos: emplear estos sustratos como fuente de energía para la síntesis de nuevas macromoléculas y deshacerse de estructuras perjudiciales para la célula (Mizushima y Klionsky, 2007). Existen tres subtipos de autofagia que se detallan en la figura 7: la macroautofagia, la microautofagia y la mediada por chaperonas (Funderburk y cols., 2010).

Fisiológicamente, existe un nivel basal de autofagia responsable del correcto recambio de proteínas y orgánulos, que es muy importante

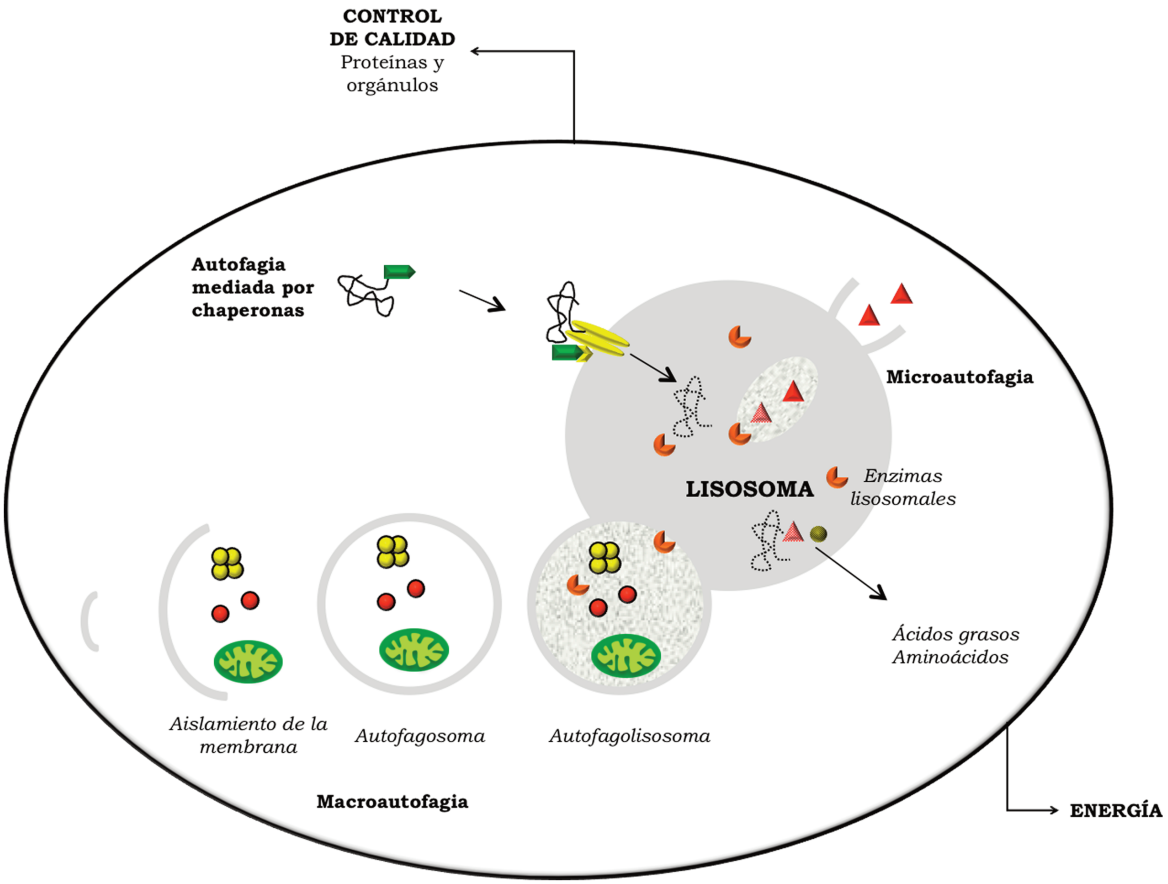

Figura 7. Subtipos de autofagia que participan en la degradación de proteínas y componentes citosólicos celulares. En la macroautofagia, regiones enteras del citosol englobando orgánulos, agregados proteicos o proteínas mal plegadas entre otros, quedan secuestradas en una vacuola de doble membrana, denominada autofagosoma, que posteriormente se fusiona con el lisosoma (Yorimitsu y Klionsky, 2005). En la microautofagia también se procesan regiones enteras del citosol, pero es la propia membrana lisosomal la que se invagina para captar los componentes citosólicos (Li y cols., 2012). La autofagia mediada por chaperonas degrada selectivamente proteínas que presentan una secuencia de aminoácidos concreta que éstas reconocen (Kaushik y cols., 2011). Los objetivos de la autofagia son dos, por un lado constituir una fuente alternativa de energía, proveyendo a la célula de aminoácidos y ácidos grasos, y por otro lado permitir la eliminación de proteínas alteradas y orgánulos dañados. Modificado de (Cuervo, 2008).

principalmente en células quiescentes como las neuronas, ya que les permite deshacerse de proteínas citosólicas aberrantes cuya acumulación podría conducir a la muerte neuronal (Hara y cols., 2006). Sin embargo, aunque la autofagia puede actuar como protector contra estímulos agresivos, también puede convertirse en un mecanismo de muerte neuronal en sí mismo. Morfológicamente, la muerte celular por autofagia se caracteriza por la ausencia de condensación de cromatina y la presencia de numerosas vacuolas en el citoplasma (Amelio y cols., 2011). En ese sentido, se ha des-

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crito la acumulación de autofagosomas disfuncionales en neuronas y neuritas distróficas de pacientes de alzhéimer (Boland y cols., 2008), que se han relacionado con la presencia de Aβ42 y con el deterioro del sistema lisosomal con la edad (Ling y Salvaterra, 2011; Soura y cols., 2012). Este elevado número de vacuolas disfuncionales podrían conducir a la muerte neuronal porque la célula no podría hacer frente a la degradación de la gran cantidad de contenido citoplasmático acumulado en las mismas (Mizushima y cols., 2008; Amelio y cols., 2011) o por daños en la membrana de los autofagosomas que liberarían su contenido al citoplasma (Ling y Salvaterra, 2009).

2.4 Pérdida sináptica

La pérdida de las sinapsis, y, por lo tanto, la pérdida de comunicación entre las neuronas, es una de las principales causas de la disfunción cognitiva en la EA (Arendt, 2009). Además de la pérdida sináptica producida por la muerte neuronal, existen evidencias de que las neuronas supervivientes también pierden sus sinapsis en la enfermedad (Coleman y Yao, 2003).

Las alteraciones de la integridad sináptica se detectan ya en pacientes con deterioro cognitivo leve (DCL), sugiriendo que puedan ser un evento temprano en la EA (Masliah y cols., 2001; Scheff y cols., 2006). La pérdida sináptica afecta fundamentalmente al hipocampo y a la corteza cerebral y se ha descrito un 18% de pérdida de las sinapsis en la región CA1 del hipocampo en DCL que puede llegar al 55% en las fases iniciales de EA (Scheff y cols., 2007).

La función de las sinapsis es transmitir la señal eléctrica presináptica mediante la liberación de neurotransmisores al espacio sináptico, que posteriormente se convierten en una señal eléctrica en la neurona post-sináptica. Las vesículas sinápticas son orgánulos encargados de almacenar los neurotransmisores en las terminales nerviosas presinápticas y una compleja maquinaria celular se encarga de transportar los neurotransmisores hacia las vesículas, liberarlas y endocitar las vesículas de nuevo para su reciclaje. Las alteraciones en el tráfico de vesículas son una de las causas más importantes de la disfunción sináptica. Ejemplos de estas alteraciones son la reducción importante de los niveles de sinaptofisina, la principal proteína de dichas vesículas, en los cerebros de pacientes de EA (Callahan y cols., 1999; Callahan y cols., 2002), los defectos en la expresión de genes relacionados con el tráfico de vesículas sinápticas (Yao y cols., 2003) o en proteínas implicadas en su recaptación, como la clatrina (Yao, 2004). Otras proteínas que se ven alteradas en la enfermedad son las que componen las membranas pre y post-sinápticas (Reddy y cols., 2005; Arendt, 2009).

En las etapas iniciales de la enfermedad se ponen en marcha mecanismos que tratan de preservar las funciones cognitivas, se incrementa la expresión

de proteínas post-sinápticas como PSD-95 (Leuba y cols., 2008) o la densidad de los botones presinápticos glutamatérgicos (Bell y cols., 2007), lo que sugiere que tiene lugar una reorganización sináptica compensatoria. Sin embargo, a medida que la enfermedad avanza, estos mecanismos se vuelven insuficientes y la pérdida sináptica se hace evidente (Arendt, 2009). De hecho, la disminución de la densidad sináptica es la alteración que mejor se correlaciona con el deterioro cognitivo en la enfermedad (DeKosky y Scheff, 1990; Coleman y Yao, 2003; Scheff y Price, 2003).

3.1 Factores genéticos

La EA es una enfermedad compleja en la que interactúan factores ambientales y genéticos. En general, se pueden distinguir dos formas principales de EA:

- EA de aparición precoz. Supone entre el 1-6% de los casos y muestra su inicio entre los 30-65 años.

- EA de aparición tardía. Supone la gran mayoría de los casos y su edad de inicio ronda los 60-65 años.

Aproximadamente en el 60% de las formas tempranas existe una historia familiar de enfermedad, y en el 13% la agregación familiar sigue un patrón de herencia autosómico dominante. El estudio de dichas familias ha permitido describir 3 genes cuyas mutaciones causan EA, se trata de los genes que codifican la proteína precursora de amiloide y las presenilinas: *APP*, *PS1* y *PS2*. Por el contrario, en el 95% de los pacientes, en los que la enfermedad aparece a partir de los 65 años, los genes implicados no son determinantes, aunque pueden conferir susceptibilidad al individuo, que a su vez puede ser modulada por factores ambientales. Estos factores, unidos a la complejidad y heterogeneidad génica que existe en la EA, hacen que hasta la fecha sólo se haya relacionado un gen de manera consistente con la EA esporádica: el gen *APOE* que codifica la apolipoproteína E. También se han presentado evidencias vinculando el

3 Factores de riesgo de la EA

riesgo de padecer EA con polimorfismos en un buen número de genes. Estos hallazgos sugieren un modelo de dicotomía genética para explicar las bases hereditarias de la enfermedad, también aplicable para otras enfermedades relacionadas con la edad como las cardiovasculares, la diabetes o el cáncer. Según este modelo, la enfermedad de aparición precoz estaría causada por una mutación rara, pero de alta penetrancia y gran impacto biológico, mientras que la forma más común de la enfermedad, de aparición tardía, está relacionada con factores genéticos de riesgo, en forma de polimorfismos comunes en la población distribuidos por todo el genoma.

3.1.1 Genes asociados con alzhéimer familiar

❍ *Mutaciones en APP*

El gen *APP* se localiza en el cromosoma 21q21. A comienzos de 2012, aparecen descritas 32 mutaciones diferentes en este gen que son capaces de producir alzhéimer en 89 familias (www.molgen.ua.ac.be/ADMutations/). Una gran parte de las mutaciones en *APP* se encuentra en residuos involucrados en el procesamiento de la proteína por las secretasas. Entre ellas, las mutaciones sin sentido "Swedish" (APPSW, APPK670N y M671L) o las mutaciones "London" (APPLON y APPV717I) son ejemplos de mutaciones que conducen a un aumento en la producción de Aβ y desarrollo de EA (Bekris y cols., 2010). Otras mutaciones (como "Arctic" o "Iowa") se encuentran en la región central de Aβ y provocan una mayor agregación del péptido (Brouwers y cols., 2008). (Ver figura 8).

❍ *Mutaciones en Presenilinas: PS1*

Las mutaciones en *PS1*, localizado en el cromosoma 14q24.2, son la principal causa de EA familiar y también las que dan lugar a las formas más severas de la enfermedad y con un inicio más precoz, hacia los 30 años de edad. Existen 185 mutaciones descritas hasta la actualidad en 405 familias (www.molgen.ua.ac.be/ADMutations/). La mayoría de las mismas son mutaciones sin sentido y producen una reducción en la actividad γ-secretasa y un aumento en el cociente Aβ42/Aβ40 (Citron y cols., 1997). Además, la reducción de la actividad γ-secretasa también puede contribuir a la patogénesis en estos pacientes por la alteración en la proteólisis de otros de sus sustratos como Notch (Shen y Kelleher, 2007), (figura 8).

❍ *Mutaciones en Presenilinas: PS2*

Al contrario que las mutaciones en el gen *PS1*, las mutaciones sin sentido en *PS2*, localizado en el cromosoma 1q42.13, son una causa rara de EA. La edad de comienzo también es más tardía (alrededor de los 50 años) y más heterogénea entre individuos con el mismo tipo de mutación (Bekris y cols., 2010). En la actualidad, se han descrito 13 mutaciones en *PS2* en 22 familias

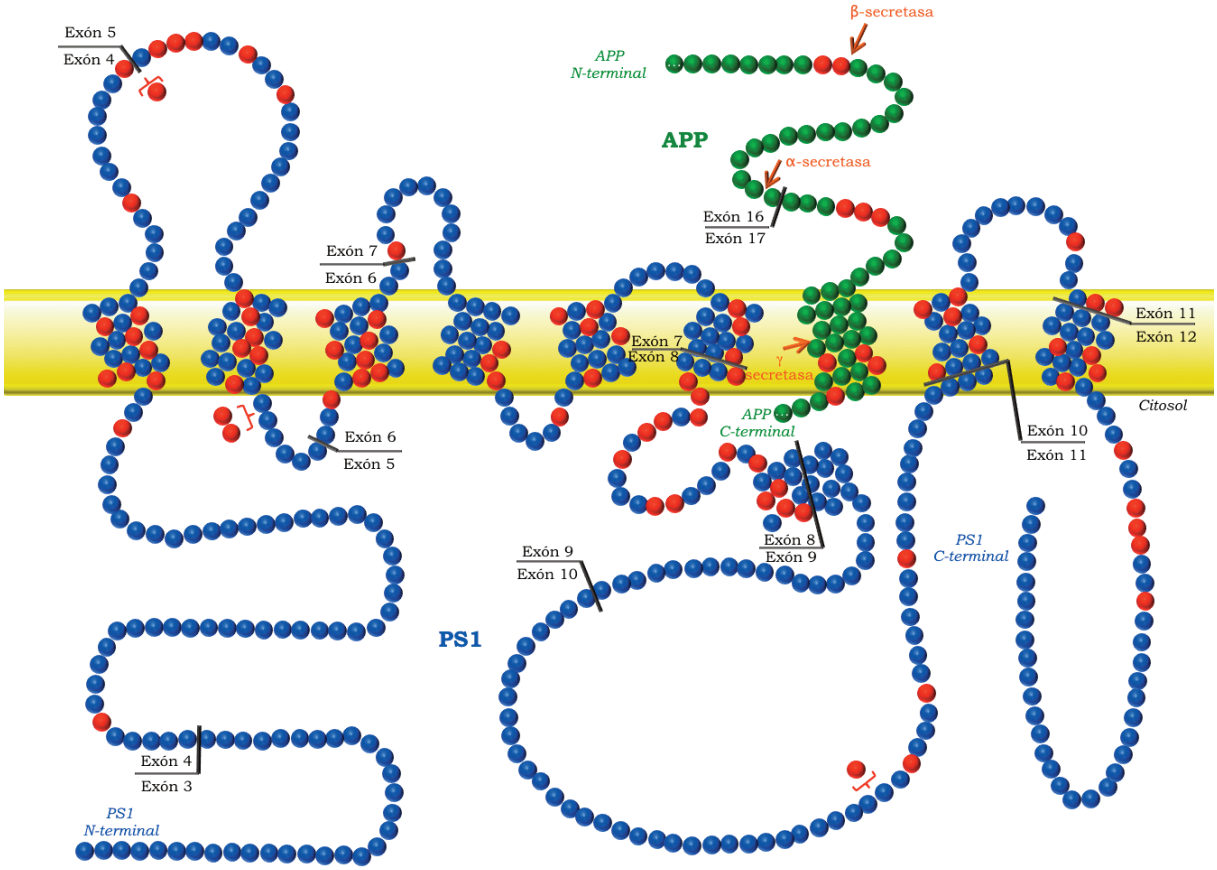

Figura 8. Esquema de las mutaciones en PS1 y APP en alzhéimer. Cada círculo representa un aminoácido. Los marcados en rojo simbolizan mutaciones sin sentido patogénicas. Los aminoácidos azules representan PS1, y los verdes APP. Se muestran también los puntos de corte de APP por las secretasas. Basado en ACS Divsion on Medicinal Chemistry (http://www.acsmedchem.org/module/AlzheimerFiles/alzheimer.html). Más información en Alzheimer Research Forum (http://www.alzforum.org).

(www.molgen.ua.ac.be/ADMutations/).

3.1.2 Genes asociados con riesgo en alzhéimer esporádico

❍ *Gen APOE*

El gen *APOE*, situado en el cromosoma 19q13.2, tiene 3 alelos principales, *APOE2*, *APOE3* y *APOE4* que dan lugar a tres isoformas de la proteína: ε2, ε3 y ε4, que se diferencian entre sí por la presencia de un aminoácido cisteína o arginina en las posiciones 112 y 158, lo que afecta a la capacidad de la proteína para unir lípidos. La variante ε2 tiene una frecuencia aproximada del 6% en la población caucásica, la ε3 del 78% y la ε4 del 16% (Seto-Salvia y Clarimon, 2010). Al comparar las frecuencias de cada una de las isoformas entre pa-

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cientes y controles por edades, existe de forma consistente un incremento del alelo ε4 en pacientes con EA esporádica respecto a controles. Respecto al genotipo ε3/ε3, el más frecuente, los heterozigotos para ε4 presentan un riesgo aproximadamente 3 veces mayor de desarrollar alzhéimer, que se eleva hasta un riesgo 15 veces mayor para los homozigotos ε4/ε4 (Ashford, 2004), de modo que la dosis génica de *APOε4* se considera un factor de riesgo para EA. Sin embargo, también hay que tener en cuenta que un 50% de las personas con al menos un alelo ε4 no desarrollan EA, o, en el sentido contrario, aproximadamente el 42% de las personas con EA esporádica no tienen un alelo *ε4*, lo que da idea de la complejidad tanto genética como ambiental que rodea a la enfermedad (Bekris y cols., 2010). Los mecanismos por los que la isoforma ε4 podría ser tóxica en cerebro no están del todo claros, pero se apunta a que pueda verse afectada la eliminación de β-amiloide a través de la barrera hematoencefálica (Jiang y cols., 2008).

❍ *Otros genes de riesgo*

Diversos estudios han mostrado hasta la fecha como polimorfismos en los genes que codifican algunas fosfoquinasas, como *DYRK1A* o *GSK3*β, capaces de fosforilar tau, están relacionados con un aumento de riesgo en EA (Ballard y cols., 2011).

Desde el 2007 ha cambiado el concepto del estudio genético de la EA gracias a nuevas técnicas de genotipado que están permitiendo realizar estudios de asociación del genoma entero, GWAS (*Genome-Wide Association Studies)* en un elevado número de pacientes y controles. Entre los genes más destacados encontrados en estos estudios hasta la fecha, merece la pena resaltar genes asociados con la inhibición del complemento y la inflamación (como *CLU*, que codifica la lipoproteína clusterina; o *CR1),* genes asociados con rutas endocíticas (como *BIN1* o *PICALM)* o con el metabolismo lipídico y de colesterol (los ya mencionados *APOE* y *CLU)* (Guerreiro y Hardy, 2011). Aunque los riesgos asociados con estos genes son pequeños, este tipo de estudios pueden ser importantes para conocer las bases moleculares de la enfermedad y poder identificar posibles dianas terapéuticas. Para más información, la base de datos www.alzgene.org proporciona un completo meta-análisis de cientos de estudios de asociación genética en alzhéimer.

3.2 Factores de riesgo no genéticos

El principal factor de riesgo para la EA es la edad avanzada (Ferri y cols., 2005). Junto con éste, los traumatismos craneales han mostrado también estar relacionados con el desarrollo de la enfermedad (Luukinen y cols., 2008; Johnson y

cols., 2010). Como ejemplo, la demencia pugilística, asociada con traumatismos craneales crónicos, se desarrolla con una neuropatología similar a la del alzhéimer: placas de Aβ, ovillos neurofibrilares, activación de la glía y neuroinflamación (Saing y cols., 2012).

Una mayor reserva cognitiva (un concepto que combina niveles de educación, tipo de ocupación y realización de actividades mentales complejas) se asocia con menor incidencia de EA, especialmente en edades más avanzadas (Valenzuela y Sachdev, 2006). Los cambios en los niveles de estrógenos tras la menopausia también se relacionan con la mayor incidencia de la enfermedad en mujeres, por la pérdida del efecto neuroprotector de los mismos (Cholerton y cols., 2002; Janicki y Schupf, 2010).

Ejemplos de factores de riesgo relacionados con el estilo de vida, y por lo tanto, modificables, son la obesidad en la mediana edad (Beydoun y cols., 2008), la falta de actividad física (Hamer y Chida, 2009) y el consumo de tabaco (Lee y cols., 2010) y alcohol (Anstey y cols., 2009).

Diversos meta-análisis de estudios observacionales han mostrado que algunas enfermedades en la mediana edad como la hipertensión (Qiu y cols., 2005), la diabetes (Lu y cols., 2009), la hipercolesterolemia (Anstey y cols., 2008) o la depresión (Ownby y cols., 2006) incrementan el riesgo de padecer la enfermedad. Una revisión reciente, calcula que la intervención en algunos de estos factores de riesgo podría prevenir alrededor de un millón de casos de alzhéimer en todo el mundo (Barnes y Yaffe, 2011).

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La patogénesis de la EA es un proceso aún no comprendido del todo. El número de hipótesis que tratan de explicar su origen es cada vez mayor, pero hasta ahora ninguna de ellas ha conseguido un consenso. Se detallan a continuación los principales mecanismos implicados.

4.1 Cascada β-Amiloide

Propuesta a principios de los años 90, la hipótesis del amiloide establece que el depósito de Aβ es el evento inicial que conduce al resto de manifestaciones de la enfermedad (Selkoe, 1991; Hardy y Higgins, 1992). La agregación de los fragmentos hidrofóbicos Aβ40 y Aβ42 desencadena la formación de placas extracelulares insolubles, que según esta hipótesis son las responsables de la muerte neuronal (Selkoe, 2001). Algunas de las evidencias en las que se basa esta teoría son las siguientes:

- Aβ es el principal componente de las placas de amiloide, que, junto con los ovillos neurofibrilares, son las dos características histopatológicas diagnósticas de la enfermedad (Zhang y cols., 2012).

- El depósito de Aβ ocurre antes que otros eventos patológicos como la formación de ovillos neurofibrilares o la muerte neuronal (Donev y cols., 2009).

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- Las personas con síndrome de Down, caracterizado por la trisomía del cromosoma 21 y que por lo tanto poseen tres copias del gen *APP*, desarrollan a edad temprana signos clínicos y patológicos de alzhéimer (Zigman y Lott, 2007; Moncaster y cols., 2010).

- Un gran porcentaje de los casos de EA de aparición precoz están producidos por mutaciones en los genes *APP*, *PS1* y *PS2* (Bekris y cols., 2010). En general, estas mutaciones conducen a un aumento en la producción de Aβ y del cociente Aβ42/Aβ40, aunque es un tema de debate si se produce un aumento tóxico o una pérdida de las funciones fisiológicas de APP y las presenilinas (De Strooper, 2007; Wolfe, 2007).

- Se han generado multitud de modelos animales de EA basados en las mutaciones de los genes *APP* y *PSEN* en el alzhéimer familiar, que desarrollan placas y muestran alteraciones en la memoria (Pimplikar, 2009).

- Aβ ha demostrado *in vitro* ser neurotóxico (Pimplikar, 2009) y producir muerte neuronal (Caughey y Lansbury, 2003). Además, induce distintos efectos citotóxicos como estrés oxidativo o alteraciones en la homeostasis del calcio, que directa o indirectamente pueden conducir a la muerte neuronal (LaFerla, 2002; Abramov y cols., 2004). También es capaz de alterar la actividad de distintas quinasas como GSK3β o Cdk5, causando hiperfosforilación de tau y formación de ovillos neurofibrilares (Lopes y cols., 2010; Cai y cols., 2012).

Aunque todas estas evidencias explican parte de los mecanismos que causan la patogénesis en alzhéimer, también existen evidencias en contra de esta hipótesis.

- Se han encontrado placas de amiloide en autopsias de personas de elevada edad sin signos de demencia (Morishima-Kawashima y cols., 2000). Además, los avances en las técnicas de neuroimagen *in vivo* (como la retención de ^{11}C-PiB (*^{11}C-labelled Pittsburgh compound B*), que se emplea para visualizar Aβ) también han mostrado depósitos en personas cognitivamente normales (Nordberg, 2008; Villemagne y cols., 2008).

- Algunos modelos de ratón muestran déficits de memoria mucho antes de que las placas aparezcan en el cerebro (Lesne y cols., 2008).

- Las mutaciones en *PS1*, que causan la gran mayoría de los casos de EA familiar, tienen efectos neurotóxicos que son independientes de la función γ-secretasa de la presenilina en el procesamiento de APP y la producción de Aβ (Baki y cols., 2004; Shen y Kelleher, 2007; Kallhoff-Munoz y cols., 2008).

- A pesar de ser una característica histopatológica importante de la enfermedad, la cantidad de depósitos de Aβ no se correlaciona con el grado de demencia (Terry y cols., 1991; Dickson y cols., 1995).

En este sentido, en los últimos años se ha descrito que son los niveles de las formas solubles de Aβ y no los insolubles los que mejor correlacionan con la severidad de la enfermedad (McLean y cols., 1999; Wang y cols., 1999), sugiriendo que las formas solubles podrían tener un papel más importante en la enfermedad que los depósitos.

Estudios posteriores han mostrado los efectos neurotóxicos de estos oligómeros solubles (Selkoe, 2008; Lublin y Gandy, 2010; Larson y Lesne, 2012), lo que ha hecho pensar que quizá las placas insolubles no sean las que desencadenan las alteraciones patológicas, sino que sean benignas o incluso protectoras frente a la toxicidad de las formas solubles (Caughey y Lansbury, 2003).

Aunque el papel de Aβ sea más limitado de lo que se creía en un principio, no hay duda de que es uno de los factores que influyen en la patogénesis de la enfermedad.

4.2 Tau

Aunque durante mucho tiempo la hipótesis de β-amiloide dominó la investigación sobre EA, la importancia de tau poco a poco ha empezado a ganar peso (Small y Duff, 2008).

Los ovillos neurofibrilares, formados principalmente por tau, son también una característica histopatológica diagnóstica de la enfermedad, pero, al contrario que las placas de amiloide, éstos sí que se correlacionan en número y localización con el grado de severidad de la EA (Giannakopoulos y cols., 2003), lo que ha hecho pensar que puedan contribuir a la muerte neuronal (Spires-Jones y cols., 2011). A pesar de ello, otros estudios señalan que la pérdida neuronal excede en número a los ovillos, y que en algunas regiones cerebrales ambas características no se correlacionan (Gomez-Isla y cols., 1997; van de Nes y cols., 2008).

La función principal de tau es la estabilización de los microtúbulos, esenciales para el transporte de vesículas y orgánulos entre el soma y las neuritas (Caviston y Holzbaur, 2006). Cuando tau se hiperfosforila, se desliga de los microtúbulos y se transloca al compartimento somatodendrítico, donde sigue fosforilándose y termina formando los ovillos neurofibrilares (Honson y Kuret, 2008). La deslocalización de tau altera por lo tanto la función de los microtúbulos, afectando entre otras cosas al transporte de vesículas y orgánulos del soma a los axones (Stamer y cols., 2002; Muresan y Muresan, 2009). Las mitocondrias, encargadas del suministro de energía y la homeostasis del calcio, son las principales afectadas, y su depleción en los axones puede ser clave en el proceso de neurodegeneración (Pritchard y cols., 2011).

Tau también es un mediador de la toxicidad inducida por Aβ (Ferrari y cols., 2003). Se ha descrito que las neuronas de ratones tau$^{-/-}$ son resistentes a la degeneración de las neuritas me-

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diada por Aβ (Rapoport y cols., 2002), o que la reducción de los niveles endógenos de tau de un modelo APP de ratón al cruzarlo con un tau$^{-/-}$, mejoraba el déficit cognitivo sin alterar los niveles de Aβ (Roberson y cols., 2007) . Aunque estos datos indican que tau actuaría por debajo de Aβ, las mutaciones en tau son la causa de enfermedades neurodegenerativas como la demencia frontotemporal con parkinsonismo asociada al cromosoma 17, en la que no hay patología mediada por Aβ (Lee y cols., 2001).

Paralelamente a lo que sucede con las placas de amiloide, la toxicidad de tau estaría mediada por las formas no-agregadas, no por los ovillos neurofibrilares (Hernandez y Avila, 2008). Recientemente se ha establecido un nuevo modelo en el que se observa activación de caspasas previa a los depósitos de tau. Al activarse, las caspasas procesan tau, y ésta polimeriza formando ovillos que también engloban a la proteína completa, lo que indicaría que en lugar de ser una causa, los depósitos de tau son una consecuencia del proceso neurodegenerativo marcado por la activación de las caspasas. Los autores además indican que las neuronas no mueren inmediatamente tras la activación de las caspasas, ya que la apoptosis no se completa, sugiriendo que los ovillos pueden ser un elemento protector (Avila, 2010; de Calignon y cols., 2010).

4.3 Inflamación

Esta hipótesis establece que la inflamación contribuye significativamente a la patogénesis en alzhéimer (McGeer y cols., 2000). En la EA, la respuesta inflamatoria es local e innata, y aunque en un inicio puede ser beneficiosa, cuando es excesiva o no termina de una manera correcta, se vuelve crónica y puede causar alteraciones neuronales (Hoozemans y cols., 2011). En el cerebro, los procesos inflamatorios están dirigidos por células de la glía: la microglía y los astrocitos.

La ***microglía*** constituye el 10% de las células en el sistema nervioso y representa la respuesta inmune innata en el cerebro, la primera línea de defensa ante estímulos patogénicos (Ransohoff y Brown, 2012). En condiciones patológicas, estas células se activan, migran, rodean las células muertas o dañadas y fagocitan los restos, de manera similar a los macrófagos (Heneka y cols., 2010). En pacientes de EA se ha observado activación de la microglía desde etapas tempranas (Cagnin y cols., 2001) y numerosos estudios apuntan a que su activación se produce en respuesta al depósito de Aβ (Barger y Harmon, 1997; DeGiorgio y cols., 2002). De hecho, las placas de amiloide se encuentran asociadas con astrocitos reactivos, microglía activada y marcadores de neuroinflamación, revisado en (Hensley, 2010). Al mismo tiempo, los mediadores de la inflamación pueden incrementar la producción de Aβ, retroalimentando el ciclo (Sastre y cols., 2008). En sentido con-

trario, en etapas muy tempranas de la enfermedad, la activación de la microglía puede reducir la acumulación de Aβ, aumentando su fagocitosis y favoreciendo su degradación (Frautschy y cols., 1998).

Estudios recientes también apuntan a que caspasas implicadas en apoptosis como la 8 y 3/7, son capaces de activar la microglía en ausencia de muerte neuronal (Burguillos y cols., 2011; Venero y cols., 2011). La activación de la microglía se desarrolla con un aumento en el número de células y cambios morfológicos, que suponen pasar de la morfología en reposo a una morfología activada caracterizada por presentar un pequeño soma y múltiples ramificaciones (Kettenmann y cols., 2011), (figura 9). Además, la activación de la microglía supone la secreción de mediadores de la inflamación como citoquinas, quimioquinas, prostaglandinas, derivados del óxido nítrico, ciclooxigenasa 2 (COX-2) o radicales libres (Ransohoff y Perry, 2009). Muchos de estos mediadores, como IL-1β, IL-6, TNF-α o TGF-β *(Transforming Growth Factor* β*)* aparecen elevados en EA (Sastre y cols., 2006b) y la producción sostenida de estas moléculas resulta neurotóxica, revisado en (Block y cols., 2007).

Por su parte, los ***astrocitos***, las células más abundantes de la glía, son células no neuronales del sistema nervioso que proporcionan soporte y protección a las neuronas, regulando su homeostasis (Li y cols., 2011). Los astrocitos se activan en respuesta a un daño en un proceso denominado astrogliosis reactiva, que se caracteriza por la proliferación de los astrocitos, cambios morfológicos en los que exitienden sus procesos y la elevada expresión de algunas proteínas, como la proteína fibrilar ácidica glial GFAP (*Glial Fibrillary Acidic Protein*) que les permiten llegar a la zona dañada (Sofroniew y Vinters, 2010). En casos severos, la astrogliosis reactiva puede acabar formando lo que se conoce como cicatriz glial, una acumulación de astrocitos reactivos rodeados de tejido conjuntivo, que separa las neuronas antes conectadas e impide el restablecimiento de nuevas conexiones neuronales (Sofroniew, 2009).

En respuesta a distintos estímulos, los astrocitos reactivos secretan moléculas proinflamatorias y quimioquinas, como IL-1β, IL-6, TNFα o TGF, que pueden estar implicadas en los procesos neuroinflamatorios de EA (revisado en Li y cols., 2011).

En la EA, los astrocitos suelen aparecer rodeando las placas de amiloide, y formando pequeñas cicatrices alrededor de las mismas que podrían actuar como barreras neuroprotectoras (Sofroniew y Vinters, 2010). Además, son cruciales para la degradación de los depósitos de Aβ (Wyss-Coray y cols., 2003) en un proceso mediado por ApoE, presente de forma muy importante en los astrocitos (Koistinaho y cols., 2004).

También existen evidencias genéticas que

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apoyan el papel de la inflamación en el desarrollo de la enfermedad. Dos recientes estudios de asociación del genoma identificaron dos genes asociados con un mayor riesgo de desarrollar EA: *CLU* y *CR1*, que codifican clusterina y el receptor 1 del complemento respectivamente, ambas implicadas en la inflamación (Guerreiro y Hardy, 2011).

Por todos estos motivos, la inflamación se ha convertido en un importante objetivo de los estudios de tratamientos farmacológicos contra la EA. Entre las primeras observaciones que se tuvieron en cuenta, está la baja prevalencia de EA en pacientes con artritis reumatoide, que algunos autores asociaron al tratamiento crónico de estos pacientes con antiinflamatorios (Hoozemans y cols., 2011). Desde entones, al menos 30 estudios epidemiológicos han estudiado la relación entre los antiinflamatorios no esteroideos (AINEs), y el riesgo de desarrollar EA. Estos estudios observa-

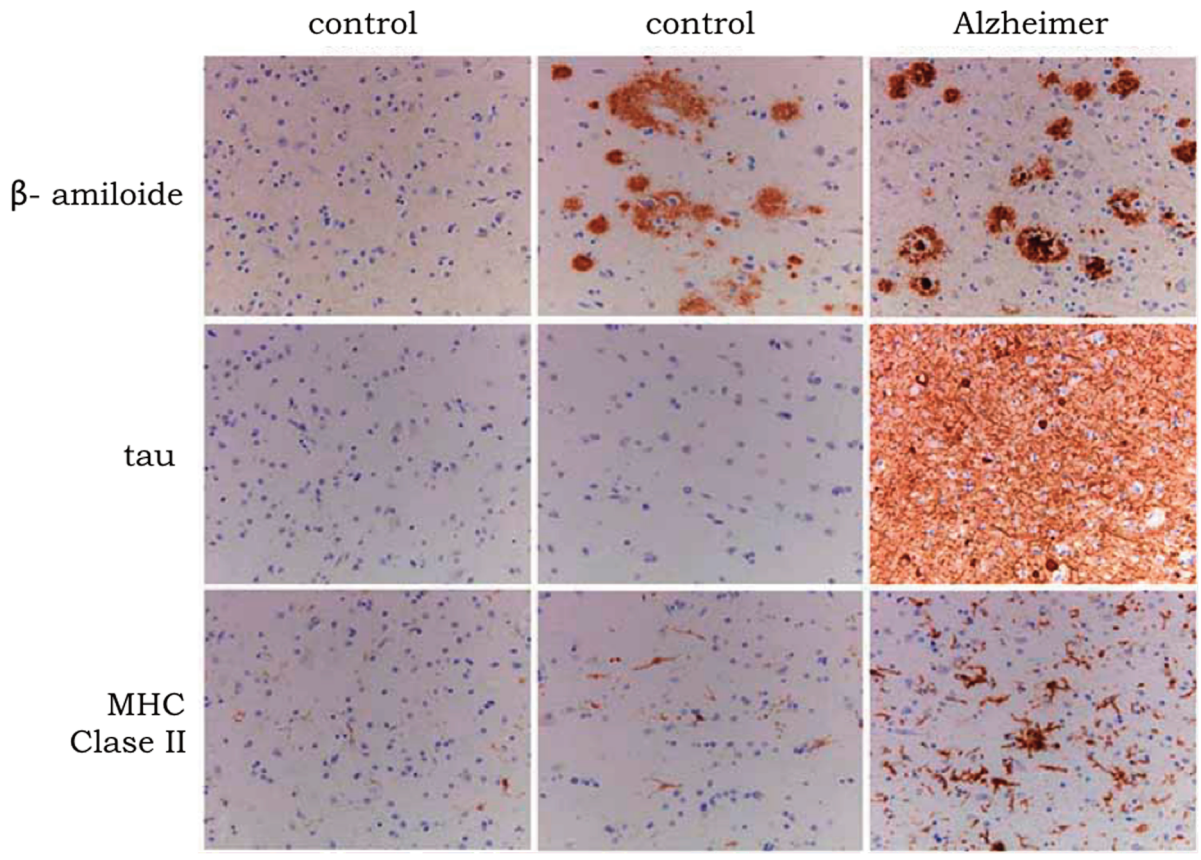

Figura 9. Microglía, amiloide y tau. La activación de la microglía (detectada con MHC II) se relaciona con los depósitos de Aβ. Mientras en controles con presencia de placas difusas de Aβ la activación de la microglía es levemente superior a la de los controles sin placas, en pacientes con las típicas placas neuríticas, la activación de la microglía es claramente mayor. Ni las placas difusas ni la activación de la microglía parecen estar relacionadas con tau fosforilada. Tomada de (Hoozemans y cols., 2011).

cionales apuntan a un retraso en la aparición de EA con el uso de AINEs (Breitner y cols., 2009) y a la disminución del riesgo de desarrollar la enfermedad (int' Veld y cols., 2001). Sin embargo, en ensayos clínicos randomizados los AINEs no se muestran efectivos en pacientes con EA establecida (Pasqualetti y cols., 2009), ni en la prevención de la aparición de la enfermedad en personas con DCL (Thal y cols., 2005). En individuos sanos se comenzó un ensayo que terminó prematuramente por riesgos cardiovasculares relacionados con el antiinflamatorio, aunque los datos preliminares tampoco mostraban resultados positivos (Meinert y cols., 2009; Breitner y cols., 2011). Los ensayos clínicos realizados hasta la actualidad han sido revisados por Szekely y Zandi, 2010. Los distintos resultados entre los estudios observacionales y los ensayos clínicos hacen pensar que los AINEs sólo sean eficaces en la EA cuando se comienzan a tomar varios años antes de su inicio (Zandi y cols., 2002), (figura 10).

4.4 Estrés oxidativo

El estrés oxidativo se define como un desequilibrio entre la producción de especies reactivas de oxígeno, ROS, (*Reactive Oxygen Species)* y su eliminación. En condiciones fisiológicas, la producción de ROS es un proceso altamente controlado por antioxidantes como glutation, α-tocoferol (vitamina E), carotenoides o ácido ascórbico y por enzimas como la catalasa o la glutation peroxidasa, que convierten el H_2O_2 en H_2O y O_2 (Yu y Chung, 2006). Sin embargo, cuando los niveles de ROS sobrepasan la capacidad antioxidante de la célula, el estrés oxidativo puede ocasionar daños celulares (Yu y Chung, 2006).

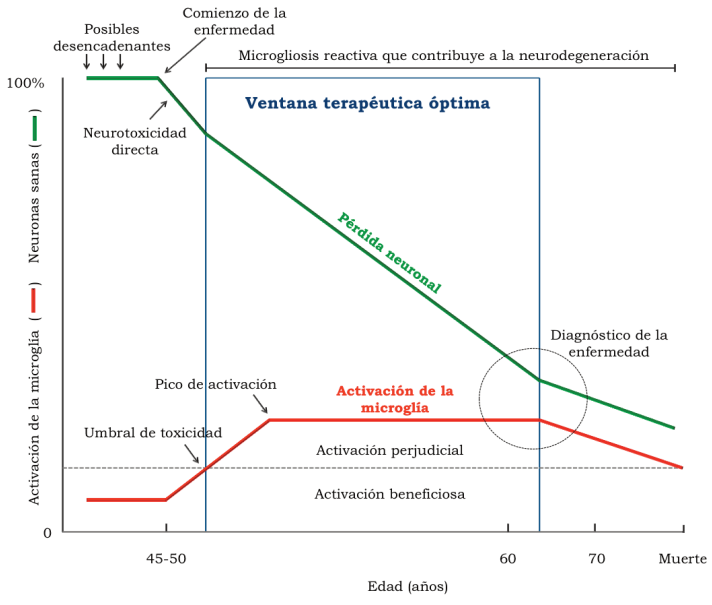

Figura 10. Relación entre activación de la microglía y muerte neuronal. El daño neuronal comienza con un estímulo que a su vez activa la microglía. Cuando la activación de la microglía supera un cierto límite, comienza a ser perjudicial para las células. Según este modelo, el diagnóstico y los tratamientos terapéuticos comienzan demasiado tarde, fuera de la ventana terapéutica óptima. El régimen terapéutico ideal empleando AINEs, comenzaría con la prevención, reduciendo la activación de la microglía hasta niveles no perjudiciales. Adaptado de (Block y cols., 2007).

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Aunque representa solamente el 2% del peso corporal, el cerebro emplea alrededor del 20% del consumo total de oxígeno del cuerpo, y, por lo tanto, genera niveles elevados de ROS, que hacen que las neuronas estén más expuestas a los radicales libres que otros sistemas celulares (Shulman y cols., 2004). Existen múltiples evidencias que indican que la sobreproducción de ROS tiene un papel muy importante en la neurodegeneración (Su y cols., 2008). Además, la presencia de daño oxidativo ya en individuos con DCL sugiere que el estrés oxidativo puede ser un acontecimiento temprano en la EA (Smith y cols., 2010). Entre otros marcadores, los pacientes con DCL muestran peroxidación lipídica y de ácidos nucleicos en cerebro, líquido cefalorraquídeo, plasma y leucocitos periféricos (Keller y cols., 2005; Bonda y cols., 2010). Por su parte, en cerebros de pacientes se ha encontrado un elevado nivel de hierro y cobre junto con oxidación del ADN, peroxidación lipídica, alteraciones mitocondriales y producción de productos de glicosilación avanzada (de la Torre, 2011).

La edad, algunas enfermedades (hipertensión, diabetes, hipercolesterolemia) o el estilo de vida (fumar, alta ingesta calórica, falta de ejercicio) contribuyen al aumento del estrés oxidativo (Maruszak y Zekanowski, 2011). El consumo de antioxidantes como la vitamina E y la C se ha asociado en algunos estudios epidemiológicos con una reducida incidencia y prevalencia de la enfermedad (Zandi y cols., 2004), aunque en otros ensayos clínicos la administración de vitamina E en pacientes con la enfermedad no muestra efectos significativamente beneficiosos (Luchsinger y cols., 2003).

4.4.1 Fuentes de estrés oxidativo en la EA

❍ *Mitocondria*

Las mitocondrias son el principal centro de actividad metabólica celular, proporcionan ATP a la célula a través del proceso de fosforilación oxidativa, lo que las convierte en grandes productoras de estrés oxidativo, pero a su vez en dianas importantes de sus efectos. Por ejemplo, el daño oxidativo en la cadena de transporte de electrones puede hacer que este complejo no transfiera los electrones de manera eficaz en el proceso de fosforilación oxidativa, generando de esta forma más ROS. El resultado es un círculo vicioso en el cual los ROS producen estrés oxidativo que a su vez produce más ROS (Bonda y cols., 2010). Múltiples evidencias apuntan a una disfunción mitocondrial con la edad y en enfermedades neurodegenerativas como el alzhéimer. Dichas alteraciones se han descrito en cerebro, fibroblastos y células sanguíneas de pacientes, así como en modelos transgénicos de EA, revisado por Maruszak y Zekanowski, 2011. Las alteraciones mitocondriales más frecuentes en la enfermedad son las deficiencias en

enzimas clave para el metabolismo oxidativo como el complejo α-cetoglutarato deshidrogenasa o el complejo piruvato deshidrogenasa, implicados en la reducción del oxígeno molecular (Su y cols., 2008). También se han descrito múltiples mutaciones en el ADN mitocondrial (algunas exclusivas de EA) en pacientes con la enfermedad, que tienen consecuencias funcionales importantes para la mitocondria (Coskun y cols., 2004). Debido a la proximidad a la cadena de trasporte de electrones, el ADN mitocondrial es más susceptible que el nuclear al daño oxidativo y su tasa de mutaciones es también mayor (Maruszak y Zekanowski, 2011). El incremento en los niveles de ROS también puede conducir a alteraciones en el número, la dinámica y la morfología de las mitocondrias, lo que altera sus funciones, entre ellas las metabólicas, y daña las neuronas (Wang y cols., 2009a). Las alteraciones del metabolismo energético como la depleción de ATP o la disminución del consumo de glucosa en regiones específicas, también son características importantes de la EA que se asocian a la disfunción mitocondrial (Ferreira y cols., 2010; Yao y Brinton, 2011).

❍ *Depósitos de β-amiloide*

Aβ ejerce parte de sus efectos neurotóxicos generando estrés oxidativo e induciendo la oxidación de distintas biomoléculas: produce peroxidación de las membranas lipídicas (Butterfield y cols., 2002), genera H_2O_2 (Manczak y cols., 2006) e hidroxinonenal (Tamagno y cols., 2003) en neuronas, además de dañar el ADN (Xu y cols., 2001) e inactivar enzimas transportadoras como la de glucosa (Guo y Mattson, 2000).

La unión de metales de transición como el hierro y el cobre a Aβ favorecen la agregación de la proteína y sus efectos pro-oxidantes (Roberts y cols., 2012). De hecho, ambos metales aparecen acumulados en las placas de amiloide (Lovell y cols., 1998). Cuando las formas oxidadas de Fe o Cu se unen a Aβ, se reducen y producen H_2O_2, que, posteriormente, en presencia de hierro oxidado, da lugar a radicales hidroxilo en la reacción de Fenton (Tabner y cols., 2002). Los radicales libres tienen como principal diana los fosfolípidos de las membranas celulares, que, a su vez, cuando se oxidan pueden favorecer la agregación de Aβ (Koppaka y cols., 2003), ver figura 11.

Algunos estudios apuntan a que el péptido Aβ secuestra los radicales libres en las neuronas vulnerables y se deposita como una medida para protegerse contra el daño oxidativo producido por los ROS (Hayashi y cols., 2007). Sin embargo, además de neuroinflamación y daños mitocondriales, los depósitos producen más generación de radicales libres (Bonda y cols., 2010).

❍ *Microglía y astrocitos activados*

Como se dijo anteriormente, tanto la mi-

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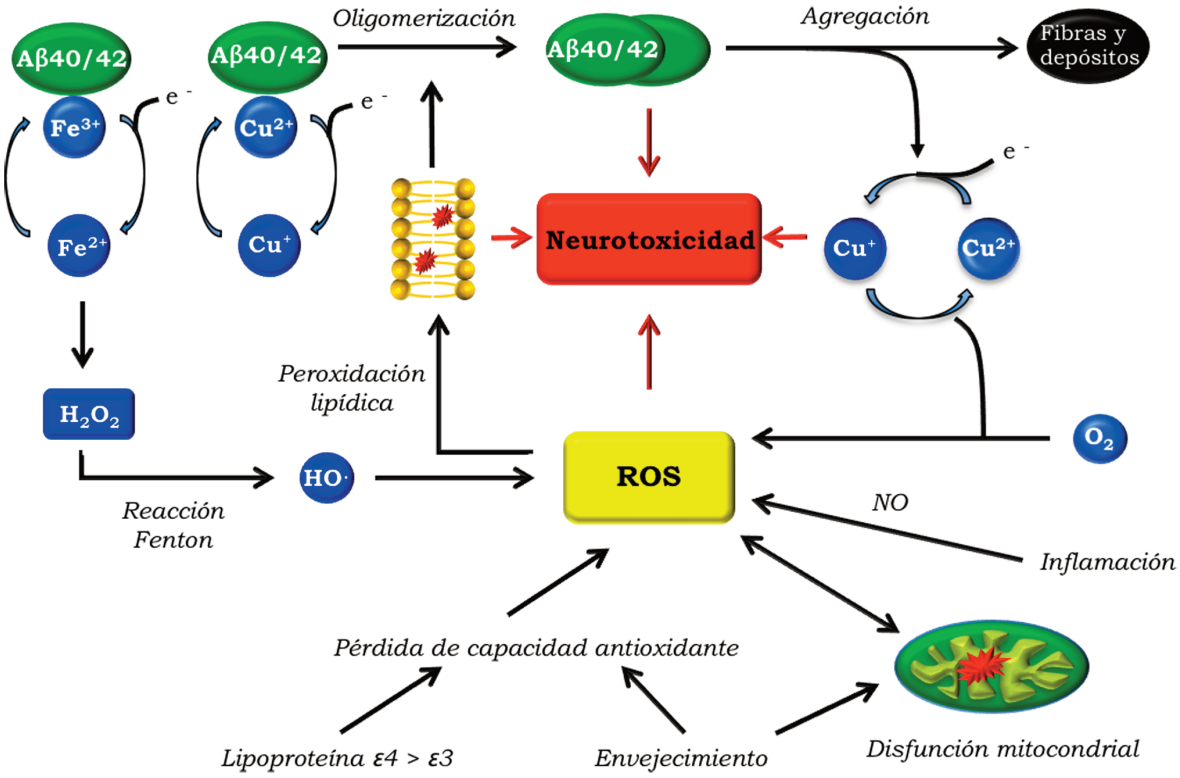

Figura 11. Interrelación entre ROS y distintos mecanismos patogénicos implicados en la EA. Basado en (Axelsen y cols., 2011).

croglía como los astrocitos activados característicos de la EA expresan numerosos mediadores de la inflamación entre los que se incluyen especies reactivas de oxígeno y de nitrógeno que les permiten atacar a los patógenos. La más característica es el óxido nítrico (NO), que induce modificaciones post-traduccionales en proteínas, como la nitración de los residuos de tirosina, y que se caracteriza por la presencia de proteínas marcadas con nitrotirosina en la EA (Smith y cols., 1997).

4.5 Homeostasis del calcio

El calcio está implicado en una enorme variedad de procesos fisiológicos, muchos de ellos neuronales, como el crecimiento y la diferenciación, la progresión del ciclo celular, la plasticidad sináptica, el aprendizaje y la memoria; y también en otros fisiopatológicos como la apoptosis y la degeneración (Bezprozvanny, 2009). Desde hace muchos años se ha observado el importante papel de la desregulación de la homeostasis del calcio en el envejecimiento y las enfermedades neurodege-

nerativas como la EA (Yu y cols., 2009a). La regulación de los niveles de calcio intracelular se produce por distintos mecanismos representados en la figura 12.

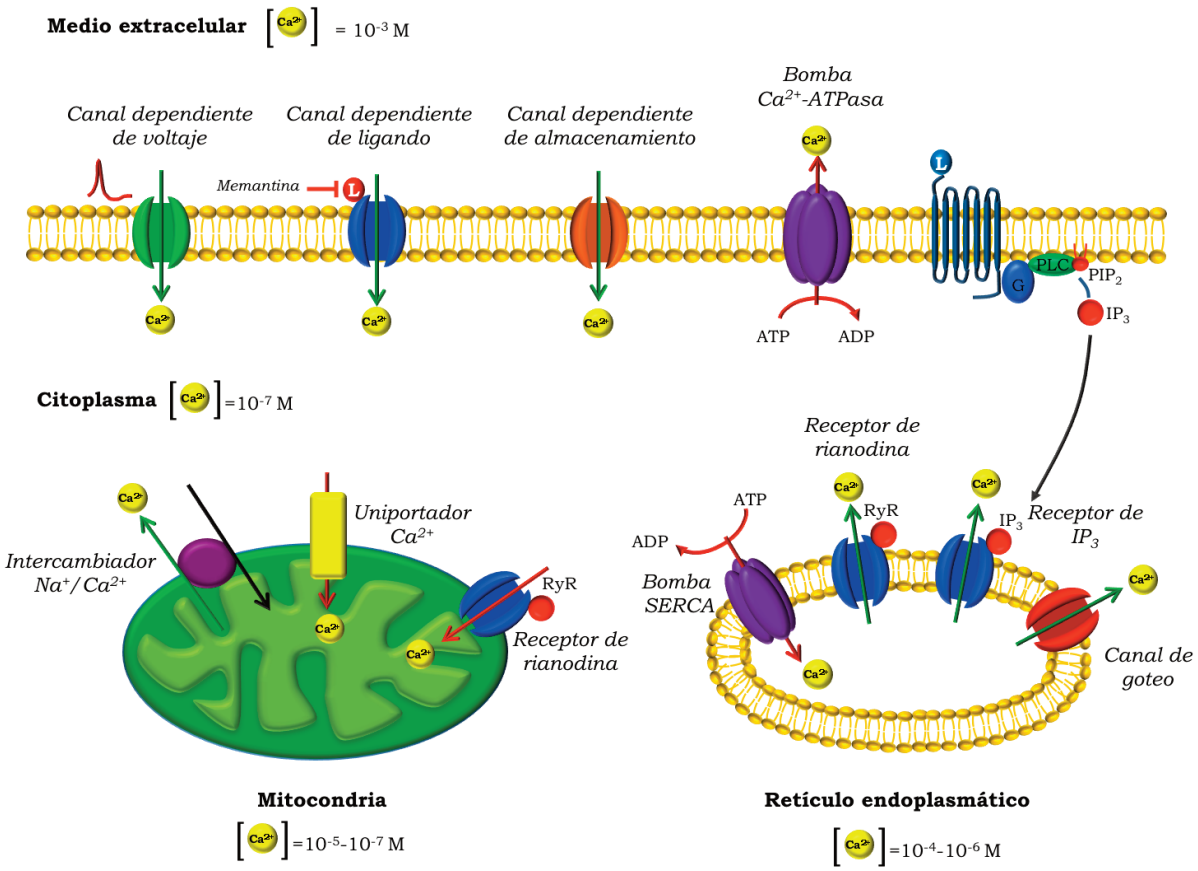

Figura 12. Regulación de la homeostasis del calcio intracelular. La concentración de calcio intracelular es varios órdenes de magnitud menor a la extracelular y la regulación de su homeostasis se produce por distintos mecanismos. El calcio entra desde el medio extracelular al citosol a través de tres tipos de canales: i)los dependientes de voltaje, que permiten la entrada de calcio tras una despolarización de la membrana; ii)los dependientes de ligando, como los asociados al receptor NMDA (N-Metil D-Aspartato) de glutamato; o iii) los dependientes de almacenamiento, que se activan por la depleción de los depósitos intracelulares. Además, el retículo endoplásmico, el principal almacén de calcio intracelular, puede liberar calcio al citosol a través de los receptores de rianodina (RyR) o de IP3, mensajero que se produce en respuesta a la activación de receptores metabotrópicos de la membrana plasmática. Las bombas de calcio Ca^{2+}-ATPasa de la membrana plasmática y SERCA (Sarco/Endoplasmic Reticulum Calcium ATPase) del retículo endoplásmico retiran el calcio del citosol y lo transportan en contra de gradiente hacia el exterior celular en el primer caso, y dentro del retículo en el segundo para mantener el equilibrio (Hermes y cols., 2010). Los canales de calcio de la mitocondria están menos caracterizados. Existe al menos un intercambiador de Na^+/Ca^{2+} que libera Ca^{2+} al citosol y un uniportador y un receptor de rianodina que introducen calcio en la mitocondria (Hoppe, 2010).

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Los sistemas de regulación del calcio experimentan alteraciones con el envejecimiento. Las consecuencias son un incremento en los niveles de calcio intracelular, mayor entrada de calcio a través de los canales dependientes de voltaje tipo L, mayor liberación desde el retículo endoplásmico a través de los receptores de IP_3 y rianodina, disminución de la capacidad tamponadora citosólica y activación de calcineurina y calpaínas (Raza y cols., 2007; Thibault y cols., 2007), proteasas implicadas en la muerte neuronal por apoptosis en la EA (Raynaud y Marcilhac, 2006).

La mitocondria también participa en la homeostasis del calcio, ya que sirve de almacén temporal en respuesta a incrementos en la concentración citosólica, por lo que las alteraciones mitocondriales relacionadas con el estrés oxidativo se relacionan también con una disfunción en la regulación del calcio (Yu y cols., 2009a). Además, si la concentración citosólica de calcio es muy elevada y la captación por la mitocondria es excesiva, se produce un aumento en la producción de ROS, se inhibe la síntesis de ATP y se libera citocromo c dando lugar a apoptosis y muerte celular (Supnet y Bezprozvanny, 2010; Rasola y Bernardi, 2011). El estrés oxidativo en la EA también se relaciona con la disfunción del calcio a través de la peroxidación de las membranas lipídicas. La generación de lípidos neurotóxicos como el 4-hidroxinonenal altera la función de proteínas de membrana implicadas en el transporte iónico como la Ca^{2+}-ATPasa o los canales dependientes de voltaje (Lu y cols., 2002).

Por su parte, las mutaciones en los genes de las presenilinas pueden alterar directamente la homeostasis del calcio, ya que interaccionan con tres componentes clave de su regulación: los receptores de IP_3, los de rianodina y las bombas SERCA (Stutzmann y cols., 2006; Cheung y cols., 2008; Green y cols., 2008). Las propias presenilinas, independientemente de su función γ-secretasa, pueden actuar como canales de calcio de goteo del retículo endoplasmático, (figura 12). Las mutaciones en *PSEN* hacen que la proteína pierda esa función y se produzca una sobrecarga de calcio en el retículo que puede desencadenar una respuesta apoptótica (Nelson y cols., 2007; Supnet y Bezprozvanny, 2011). Los productos del metabolismo de APP y Aβ pueden aumentar la concentración intracelular de calcio actuando sobre los canales o formando poros en la membrana, entre otros mecanismos, revisado en (Yu y cols., 2009a).

Además de los reservorios intracelulares como el retículo endoplásmico o la mitocondria, la célula cuenta con moléculas tamponadoras citosólicas que se unen al calcio intracelular, regulan sus niveles y están implicadas en una gran variedad de rutas de señalización (Gilabert, 2012). Entre ellas destacan la calmodulina y la calbindina, cuyas alteraciones también se han descrito en la enfermedad (McLachlan y cols., 1987; O'Day y Myre, 2004; Riascos y cols., 2011).

El calcio media el daño neuronal causado por excitotoxicidad, cuando se produce una sobreactivación de los receptores de glutamato, el principal neurotransmisor excitatorio del cerebro. La unión excesiva de glutamato al receptor NMDA conlleva un aumento extraordinario del contenido de calcio citosólico, que puede inducir apoptosis a través de calcineurina o las calpaínas, además de alterar otros orgánulos sensibles como las mitocondrias (Gazulla y Cavero-Nagore, 2006; Dong y cols., 2009). En este sentido, el antagonista de los receptores NMDA memantina, es un fármaco aprobado para el tratamiento de la EA (Lipton, 2005), ver figura 12.

4.6 Colesterol

El colesterol es un componente esencial de las membranas lipídicas de las células animales que ayuda al mantenimiento de su permeabilidad y fluidez. Además, es el principal sustrato para la síntesis de una gran variedad de esteroides, incluyendo vitaminas, ácidos biliares y hormonas. El colesterol se obtiene a través de la dieta y también se sintetiza *in vivo* en la ruta del mevalonato, donde la reacción limitante del proceso es la formación de ácido mevalónico a partir de 3-hidroxi-3-metilglutaril-Coenzima A (HMG CoA) catalizado por la HMG CoA reductasa (figura 13). Aunque el colesterol es vital para el funcionamiento celular, las alteraciones en su concentración o su homeostasis contribuyen a la aparición de enfermedades cardiovasculares y neurodegenerativas (Reiss y cols., 2004; Cui y cols., 2007). De hecho, la hipercolesterolemia es un factor de riesgo no genético para el desarrollo de la EA (Kivipelto y cols., 2001). El tratamiento con estatinas, que actúan inhibiendo la HMG CoA reductasa, se emplea para disminuir los niveles de colesterol, aunque también afecta a la síntesis de los productos derivados del mismo, como isoprenoides o ubiquinona (Nawarskas, 2005).

En el cerebro, el colesterol es un componente importante de las vainas de mielina, y de las membranas neuronales y gliales, y es fundamental para la formación de las sinapsis (Pfrieger, 2003). Las neuronas y las células gliales sintetizan su propio colesterol por la vía de la HMG-CoA reductasa, y éste forma complejos con la lipoproteína ApoE, la más abundante en cerebro, para su transporte y la formación de nuevas membranas y sinapsis (Mathew y cols., 2011). La ApoE también es encargada de transportar el exceso de colesterol al plasma para su eliminación (Bjorkhem, 2006). En la EA, se ha descrito un exceso de eliminación de colesterol debido a la degeneración de las sinapsis y también a la alteración del transporte por ApoE (Mathew y cols., 2011). Las mutaciones en ApoE se han relacionado además con un aumento del riesgo de desarrollar EA (Martin y cols., 2000; Namboori y cols., 2011).

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Además, el colesterol también se ha relacionado con la formación de Aβ en la EA debido a que el procesamiento proteolítico de APP tiene lugar en dominios de la membrana ricos en colesterol. Estudios *in vitro* muestran que cuando se trata a células humanas transfectadas con APP con inhibidores de la HMG CoA reductasa, el procesamiento de APP por la vía amiloidogénica de la β-secretasa se reduce; mientras que el tratamiento de esas células con colesterol incrementa en cuatro veces la producción de Aβ (Simons y cols., 1998). Otros estudios señalan que el colesterol se une a APP en el lugar de corte por la α-secretasa, favoreciendo el procesamiento por la β-secretasa y la formación de Aβ (Yao y Papadopoulos, 2002).

Estos resultados se correlacionan con otros estudios *in vivo* usando modelos animales. Los ratones transgénicos que sobreexpresan APP humana mostraban un incremento en la deposición de Aβ en el cerebro cuando eran sometidos a una dieta con niveles elevados colesterol (Refolo y cols., 2000; Shie y cols., 2002). Los niveles de Aβ en estos casos se correlacionaban con los niveles de colesterol en plasma. Cuando se trataba a los ratones con un inhibidor de la HMG CoA reductasa, tanto el colesterol plasmático como los depósitos de Aβ disminuían (Refolo y cols., 2001). Resultados similares se obtuvieron en conejos alimentados con un exceso de colesterol en su dieta (Sparks y cols., 1994).

Además, distintos estudios epidemiológicos muestran que los pacientes en tratamiento con estatinas presentan una menor prevalencia de EA (Jick y cols., 2000; Rockwood y cols., 2002; Shepardson y cols., 2011a). Las estatinas, además de reducir los niveles de colesterol, tienen otros efectos pleiotrópicos que no derivan de sus acciones sobre el metabolismo lipídico. Entre otras funciones, inhiben la formación de isoprenoides como el farnesilpirofosfato (FPP) o el geranilgeranilpirofosfato (GGPP), derivados del metabolismo del mevalonato (figura 13) y encargados de la modificación post-traduccional de proteínas, entre ellas pequeñas GTPasas como Rho, Rac, Rab o Ras

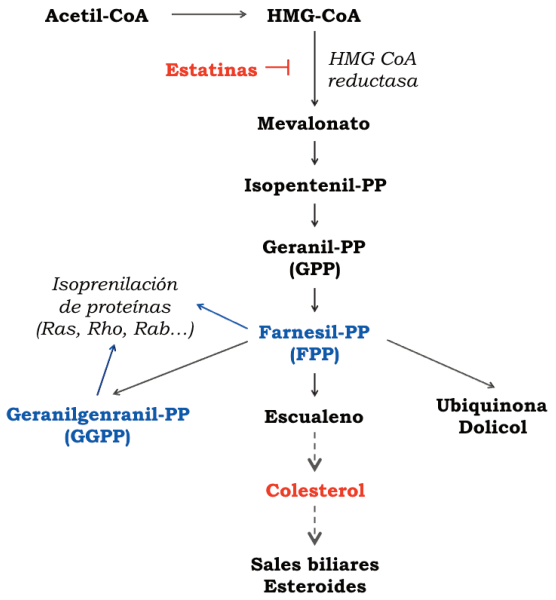

Figura 13. Esquema de la síntesis de novo del colesterol en la ruta del mevalonato. Otros productos de la ruta incluyen los isoprenoides farnesilpirofosfato (FPP) y geranilgeranilpirofosfato (GGPP). Adaptado de (Cole y Vassar, 2006) .

(Liao y Laufs, 2005). Estas moléculas también se han visto relacionadas con la patogénesis en EA, regulando la actividad de las secretasas en el metabolismo de APP (Zhou y cols., 2003; Cole y cols., 2005), la activación de la glía (Cordle y Landreth, 2005), la generación de ROS (Lee y cols., 2002) o la plasticidad sináptica (Pilpel y Segal, 2004). Además se han encontrado niveles más altos tanto de FPP como de GGPP en los cerebros de pacientes (Eckert y cols., 2009).

Por lo tanto, junto con los niveles de colesterol, también hay que tener en cuenta que la homeostasis de estos intermediarios puede ser importante en la patogénesis de la enfermedad (Cole y Vassar, 2006; Hooff y cols., 2010).

4.7 Déficit Colinérgico

La hipótesis del déficit colinérgico fue la primera que surgió para intentar explicar la patogénesis de la EA. La acetilcolina (Ach) es uno de los principales neurotransmisores en el sistema nervioso central (SNC). Se sintetiza en algunas neuronas a partir colina (Cho) y de acetil-coenzima A derivada del metabolismo de la glucosa, mediante la enzima colinacetiltransferasa (CAT). A continuación, se libera al espacio sináptico, donde se une a sus receptores, y rápidamente se degrada por medio de la acetilcolinesterasa. La colina es recaptada de nuevo por la neurona para volverse a emplear en la síntesis de Ach (Martorana y cols., 2010). La figura 14 recoge un esquema de este proceso.

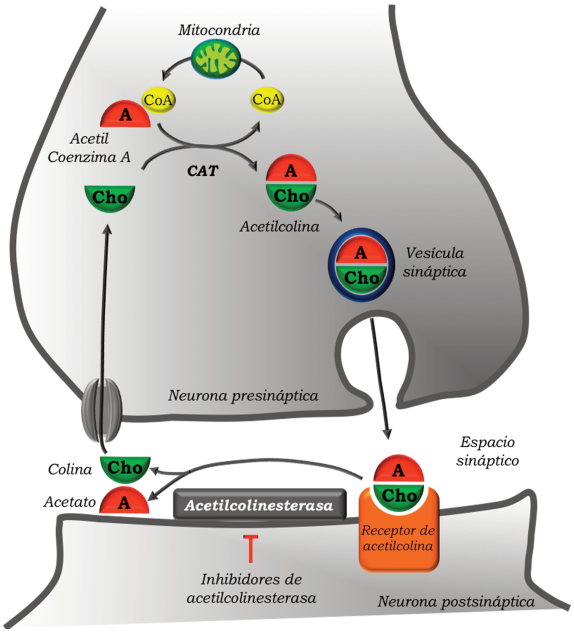

Figura 14. Esquema de la síntesis y degradación de acetilcolina en las neuronas. Basado en la página Anesthesia UK (http://www.frca.co.uk/)

A mediados de los años 70, esta hipótesis toma forma, cuando se encuentra que la actividad de la colinacetiltransferasa estaba disminuída en la corteza cerebral y el hipocampo de pacientes de alzhéimer (Perry y cols., 1977; Rossor y cols., 1982).

Posteriormente se descubrió la degeneración selectiva en la EA de las neuronas del núcleo basal de Meynert (que proyecta fibras colinérgicas hacia la corteza y el hipocampo), lo que sirvió para explicar la menor actividad de CAT en el cerebro de los pacientes (Whitehouse y cols., 1982; Mufson y cols., 2003). Éstos son eventos tempranos en

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la enfermedad (Mesulam, 2004). La acetilcolina promueve la neurogénesis en el hipocampo y está implicada en el aprendizaje, por lo tanto, el déficit colinérgico puede contribuir a las alteraciones en la memoria de los enfermos (Mohapel y cols., 2005; Veena y cols., 2011). Estas evidencias llevaron al desarrollo de fármacos que incrementaran los niveles de Ach inhibiendo la acetilcolinesterasa responsable de su degradación. En la actualidad, existen cuatro fármacos aprobados para el tratamiento del alzhéimer basados en esta hipótesis: tacrina, donepezilo, rivastigmina y galantamina, (ver figura 14) (Pepeu y Giovannini, 2009).

4.8 Degradación de proteínas: Proteasoma y Autofagia

El sistema ubiquitina-proteasoma y la autofagia son los dos principales mecanismos celulares para la degradación de proteínas. El proteasoma es un complejo enzimático por el cual las proteínas previamente marcadas con ubiquitina son procesadas y degradadas. El proteasoma tiene un papel fundamental en la eliminación de proteínas que ya no son funcionales, por ejemplo, por estar oxidadas o mal plegadas (Davies, 2001; Grune y cols., 2003; Kubota, 2009) y su inhibición conlleva la acumulación de proteínas ubiquitinadas causando muerte celular (Gorman, 2008).

En la EA se han descrito alteraciones en la funcionalidad del proteasoma (Keller y cols., 2000; Keck y cols., 2003). En concreto, el trabajo de Keller y cols. describe una disminución de su actividad en áreas afectadas selectivamente por la EA, como el hipocampo o la corteza. Las proteínas mal plegadas que escapan a la degradación suelen agregarse debido a que exponen una gran cantidad de residuos hidrofóbicos (Gorman, 2008). De modo que las alteraciones en la actividad del proteasoma podrían relacionarse con la presencia característica de agregados proteicos y proteínas oxidadas en la EA. Éstos, además, pueden exacerbar la disfunción del proteasoma (Bence y cols., 2001; Oddo, 2008). Otra evidencia de la alteración de este sistema en la EA es la acumulación de ubiquitina en las placas y los ovillos neurofibrilares (Morishima-Kawashima y cols., 1993; van Leeuwen y cols., 1998). Los propios ovillos y el péptido Aβ42 son también capaces de inhibir la actividad del proteasoma, dando lugar a un círculo vicioso que conduce a más acumulación de Aβ y tau (Keck y cols., 2003; Oh y cols., 2005).

La autofagia, implicada en la eliminación de orgánulos y proteínas agregadas a través de los lisosomas, también juega un papel importante en la homeostasis celular y la neurodegeneración, aunque el mecanismo que la relaciona con la EA no está del todo elucidado. Por un lado, como se dijo anteriormente, la acumulación de autofagosomas en las neuronas podría conducir a la muerte celular. Por otro lado, este sistema está implicado en la degradación de tau y Aβ (Wang y cols.,

2009b; Jaeger y cols., 2010), por lo tanto la disminución de la actividad autofágica puede conducir a la acumulación de dichas proteínas (Li y cols., 2010).

4.9 Ciclo celular

La hipótesis del ciclo celular surgió hace relativamente pocos años, pero son cada vez más las evidencias que apoyan que las alteraciones en el control del ciclo celular en poblaciones neuronales vulnerables del cerebro son de gran importancia en la patogénesis de la enfermedad. La teoría del ciclo celular establece que la reactivación del ciclo en neuronas maduras conduce en último término a la muerte neuronal y al desarrollo de la EA (Nagy y cols., 1998; Arendt y cols., 2000; Copani y cols., 2001; Herrup y cols., 2004).

El ciclo celular es un proceso altamente regulado que consta de cuatro fases necesarias para la división celular: G_1, S, G_2 y M. La mayoría de las células completan el ciclo entre 40 y 60 veces en su vida. Durante el desarrollo del sistema nervioso, los precursores neuronales también proliferan y las completan, sin embargo, cuando están totalmente diferenciadas, la gran mayoría de las neuronas salen del ciclo celular y permanecen en una etapa post-mitótica o quiescente, denominada fase G_0, en la que no se replican (Galderisi y cols., 2003; Politis y cols., 2008). Probablemente, las neuronas maduras tienen que mantenerse en esta fase porque una hipotética división celular conllevaría cambios en su citoesqueleto para prepararse para la mitosis y la citocinesis que alterarían las conexiones sinápticas y la función neuronal (Currais y cols., 2009). Si por alguna razón son forzadas a entrar en ciclo, la incapacidad de las neuronas diferenciadas para completarlo desencadena un proceso apoptótico que termina con la muerte neuronal y que contribuye a la patogénesis en el alzhéimer (Yang y cols., 2003; McShea y cols., 2007; Lopes y cols., 2009b).

4.9.1 Fases del ciclo celular

Para progresar a través de las distintas fases del ciclo, las células emplean ciclinas y quinasas dependientes de ciclinas (Cdks, *Cyclin-Dependent Kinase*) que alternativamente se expresan o se suprimen en función de la etapa. Este mecanismo está controlado por el sistema ubiquitina-proteasoma responsable de la proteólisis regulada de las ciclinas y de los inhibidores de las mismas (Udvardy, 1996).

Durante la fase G_1, del inglés *Growth* o *Gap1*, (ver figura 15), señales mitogénicas como factores de crecimiento, desencadenan la activación de la ciclina D, cuya unión a Cdk4 o Cdk6 les permite fosforilar la proteína del retinoblastoma (Rb) impidiendo así su unión al factor de transcripción E2F-1. Cuando éste es liberado, media la transcripción de genes que codifican proteínas que

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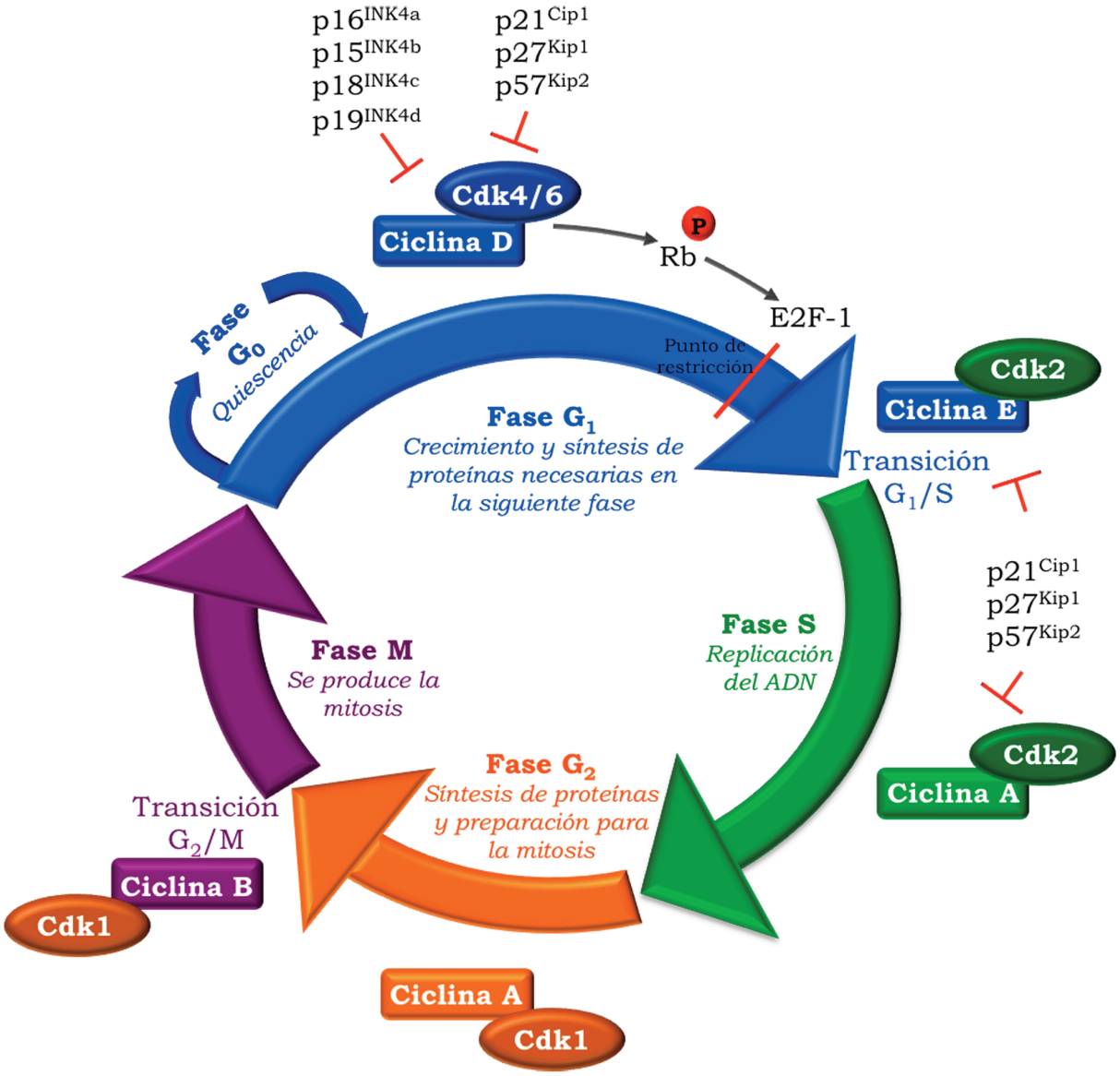

Figura 15. Esquema representativo de la progresión y la regulación del ciclo celular en eucariotas.

serán necesarias en la siguiente etapa. Al final de la fase G_1, es necesario un incremento en la actividad ciclina E/Cdk2 para asegurar la progresión hacia la siguiente fase. Al final de G_1 se encuentra el punto de restricción más importante del ciclo. Una vez que éste se supera, la entrada en ciclo es irreversible.

A lo largo de la fase S, de *Synthesis*, el complejo ciclina A-Cdk2 fosforila sustratos que permiten la replicación del ADN, y, como resultado, cada cromosoma se duplica. A continuación, comienza la fase G_2, *Growth* o *Gap2*. Este proceso, regulado por ciclina A/Cdk1 regula la fosforilación de proteínas esenciales en esta fase, en la que generalmente la célula se asegura de que la replicación del ADN ha finalizado correctamente. El complejo ciclina B/Cdk1 se encarga de permitir la progresión a la fase M, de *Mitosis*, en la que las células físicamente se dividen dando lugar a dos células hijas (Murray, 2004; Currais y cols., 2009). La actividad de las Cdks que controlan el proceso se regula también con inhibidores específicos que se organizan en dos familias, INK4 y Cip/Kip. La familia INK4 (*Inhibitors of Cyclin D-dependent Kinase 4*) está formada por $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ y $p19^{INK4d}$. Por su parte, la familia Cip/Kip (*Cdk Interacting Protein/Kinase Inhibitory Protein*) está formada por $p21^{Cip1}$, $p27^{Kip1}$ y $p57^{Kip2}$ (Currais y cols., 2009). Además, existen dos puntos importantes de control del ciclo en las transiciones G_1/S y G_2/M en los que la célula comprueba si la fase anterior se ha completado correctamente antes de pasar a la siguiente. En el primer punto de control, la célula decide si está preparada para comenzar la división celular o regresa a la fase G_0 y se regula fundamentalmente por el inhibidor de Cdk4, p16. En el segundo punto de control el principal problema suelen ser daños en el ADN. La célula debe decidir si continúa con la progresión del ciclo, si se produce un arresto para poner en marcha mecanismos de reparación o si, en el caso de que no sea posible la reparación, se induce la apoptosis (Csikasz-Nagy y cols., 2011).

4.9.2 Marcadores del ciclo celular en EA

Las neuronas de los pacientes de EA muestran un elevado número de marcadores del ciclo celular. En particular destaca la presencia de ciclina D, Cdk4 y Ki67, que indican que las neuronas han salido de la fase G_0 y han entrado en la fase G_1 (Smith y Lippa, 1995; McShea y cols., 1997; Nagy y cols., 1997b; Busser y cols., 1998; Smith y cols., 1999) (figura 16). Además, se han encontrado elevados niveles de Rb hiperfosforilada y localización citoplasmática de E2F-1 (Ranganathan y cols., 2001; Thakur y cols., 2008), así como de p130, una proteína relacionada con pRb (Previll y cols., 2007). Los inhibidores de Cdks como p16, p15, p18 y p19 también aparecen incrementados en la corteza temporal y las neuronas piramidales del hipocampo de los pacientes de EA (Arendt y cols.,

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1996; McShea y cols., 1997; Arendt y cols., 1998), así como los niveles citoplasmáticos de p27 (Ogawa y cols., 2003a). El incremento en los inhibidores de Cdks podría ser un mecanismo de defensa contra la activación de los iniciadores del ciclo (Currais y cols., 2009).

También hay evidencias de transición a la fase S, como indica la presencia de proteínas de esa fase como pmcm2 (Bonda y cols., 2009), o más importante, evidencias de duplicación del ADN que demuestran que la neurona es capaz de completar la fase S (ver figura 17). El laboratorio de Herrup, empleando técnicas de hibridación *in situ* con sondas fluorescentes, demostró que una fracción importante de neuronas piramidales del hipocampo en EA, había replicado cuatro loci genéticos en tres cromosomas diferentes (Yang y cols., 2001). El grupo de Arendt, (Mosch y cols., 2007; Arendt y cols., 2009), también cuantificó la cantidad de ADN en neuronas corticales de la enfermedad, encontrando una población de neuronas tetraploides, positivas para ciclina B1, que habían

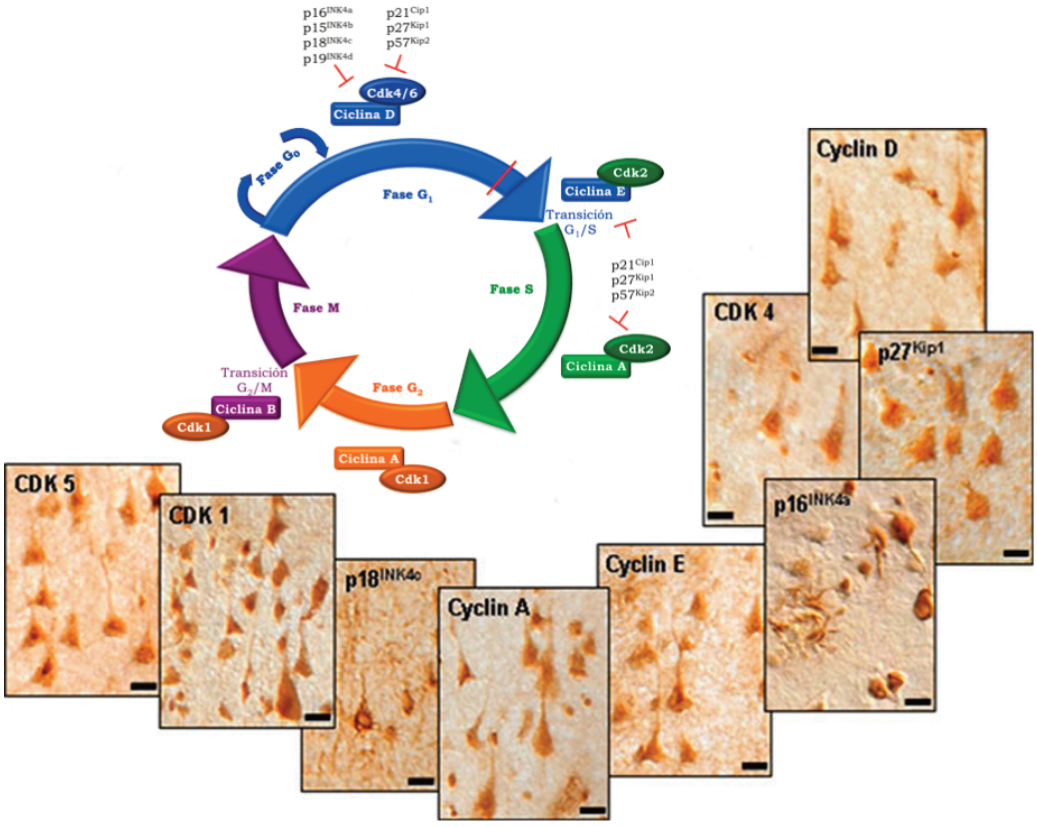

Figura 16. Marcadores del ciclo celular en el cerebro de pacientes de EA. Los principales reguladores del ciclo celular se expresan en las neuronas piramidales de las zonas afectadas en la EA antes de la neurodegeneración. Adaptado de (Arendt, 2012)

completado con éxito la replicación del ADN. Diferentes estudios han mostrado que, en condiciones fisiológicas, en el cerebro existe una pequeña tasa de neuronas aneuploides, aproximadamente del 10%, que son bien toleradas (Yurov y cols., 2005; Mosch y cols., 2007). Sin embargo, en la EA, esta frecuencia se duplica (Arendt, 2012) y el exceso hace que las neuronas con un número de cromosomas superior al diploide mueran selectivamente en etapas tempranas de la enfermedad (Arendt y cols., 2010). La tetraploidía inducida por la reactivación del ciclo se correlaciona también con cambios morfológicos e hipertrofia neuronal que pueden ser responsables de las alteraciones funcionales y los cambios en los circuitos neuronales que conducen a la neurodegeneración (Frade y Lopez-Sanchez, 2010). Algunos autores defienden que en lugar de un intento de entrada en ciclo de las neuronas maduras, la aneuploidía se debe a deficiencias en la segregación cromosómica durante el desarrollo embrionario, que dan lugar a progenitores aneuploides que no se eliminan por apoptosis como sucede en condiciones normales (Zekanowski y Wojda, 2009; Iourov y cols., 2011).

Algunas de las neuronas de las zonas afectadas progresan y se detienen en la siguiente fase del ciclo, G_2, como demuestra la presencia ciclina B en las neuronas del hipocampo (Nagy y cols., 1997a; Vincent y cols., 1997) o del regulador de la transición G_2/M CARB (*CIP-1-associated regulator of cyclin B*) (Zhu y cols., 2004a). Sin embargo, nunca se ha visto progresión a la fase M (Ogawa y cols., 2003b). A pesar de que recientemente se han encontrado neuronas binucleadas (Zhu y cols., 2008), nunca se ha descrito condensación de cromosomas, presencia del huso mitótico o de citocinesis, lo que puede indicar que las neuronas susceptibles quedan arrestadas en la transición G_2/M antes de morir. Por lo tanto, la activación de Cdk1 (Cdc2) en la fase G_2 podría ser el paso limitante previo a la apoptosis, ya que esta quinasa puede iniciar el proceso fosforilando la proteína proapoptótica BAD (Konishi y cols., 2002; Konishi y Bonni, 2003). Cdk1 se encuentra sobreexpresado en EA (Vincent y cols., 1997) y se localiza en la glía y los ovillos neurofibrilares. Sería razonable pensar que la apoptosis neuronal en G_2 puede ser el resultado de una incapacidad del citoesqueleto altamente especializado de las neuronas para formar el huso mitótico y la citocinesis (Currais y cols., 2009).

La presencia de marcadores del ciclo celular también se ha observado en personas con DCL (Sultana y Butterfield, 2007; Keeney y cols., 2011) y, en modelos animales, es previa a la aparición de otros signos neuropatológicos de la enfermedad como las placas o la inflamación (Yang y cols., 2006; Varvel y cols., 2009), lo que hace pensar que las alteraciones en el ciclo celular son un evento temprano en la patogénesis de la enfermedad.

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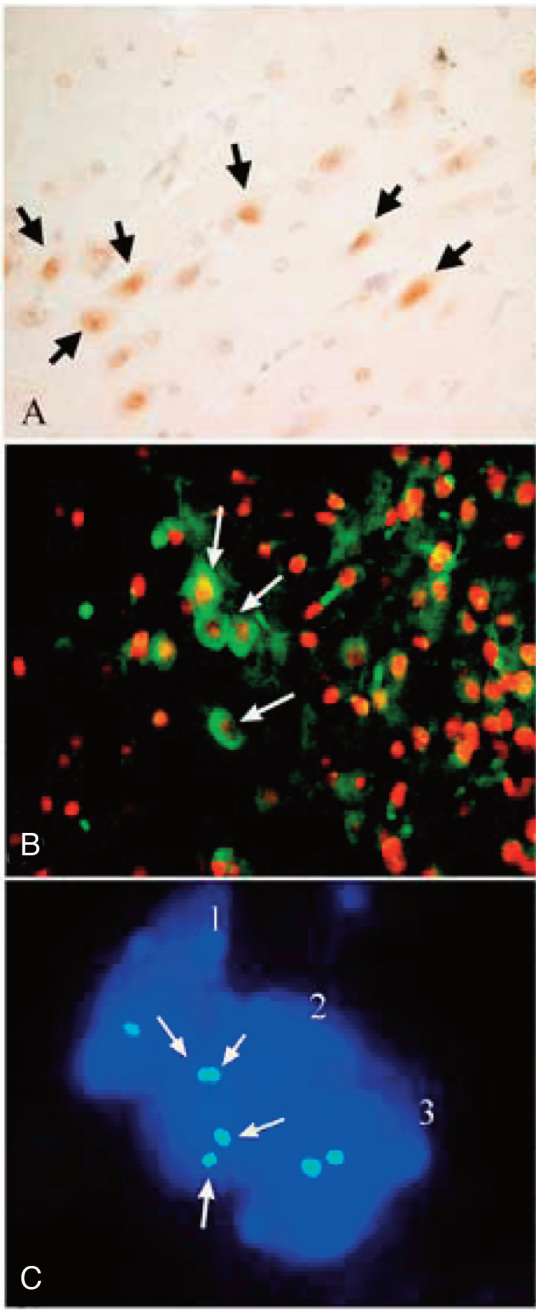

Figura 17. Alteraciones del ciclo celular en EA. A) Neuronas del hipocampo de pacientes de EA positivas para ciclina B. B) Ciclina A en nueronas corticales del modelo transgénico de EA R1.40. C) Análisis FISH en el que se muestran 4 puntos de hibridación de un único locus del genoma del ratón que indican que se ha producido la replicación del ADN en las neuronas del hipocampo del ratón R1.40. Adaptado de (Yang y Herrup, 2007)

4.9.3 Señales mitóticas

Las señales mitóticas que inducen la entrada de las neuronas en ciclo pueden ser de diversos tipos. Existen numerosos compuestos con actividad neurotrófica y potencialmente mitogénicos que se encuentran elevados en EA. Entre ellos destacan el factor de crecimiento nervioso, NGF (*Nerve Growth Factor)*, la hormona luteinizante, el factor de crecimiento epidérmico, EGF *(Epidermal Growth Factor)*, el factor de crecimiento derivado de plaquetas, PDGF *(Platelet-Derived Growth Factor)* o el factor de crecimiento transformante β1, TGF-β1, revisado en (Zhu y cols., 2004b). Algunos estudios en modelos celulares han mostrado que las neuronas pueden entrar en ciclo en respuesta a situaciones de estrés como la privación de factores tróficos, daños en el ADN o apoptosis mediada por estrés oxidativo con peróxido de hidrógeno (Herrup y Busser, 1995; Shirvan y cols., 1998; Kruman y cols., 2004).

La mayoría de estos factores median sus efectos celulares a través de la activación de la vía de las MAP (*Mitogen Activated Protein)* quinasas, una ruta dependiente de la proteína G Ras (Chang y cols., 2003). El incremento en fases muy tempranas de Ras en el cerebro de los pacientes (Gartner y cols., 1999; McShea y cols., 2007), puede ser una de las señales mitóticas que desencadena el proceso, ya que está implicada en la transición G_0/G_1 a través de su interacción con la ciclina D1

(Lavoie y cols., 1996). El resto de mediadores de la cascada de Ras (Raf, MEK1/2 y ERK1/2 *(Extracellular-signal Regulated Kinase)*) también están activados en neuronas de EA, pero no en controles sanos (Ferrer y cols., 2001; Pei y cols., 2002).

Por otra parte, tanto APP como Aβ han mostrado ser capaces de inducir la entrada en ciclo, (Schubert y cols., 1989; Varvel y cols., 2008; Nizzari y cols., 2012). Aβ puede mediar la entrada en ciclo a través de Cdk5, una quinasa también implicada en la hiperfosforilación de tau (Lopes y cols., 2010). En cultivos neuronales se ha observado como los niveles de Cdk4 (transición G_0/G_1) y PCNA (*Proliferating Cell Nuclear Antigen)* (fase S) se incrementaban en respuesta a inyecciones de Aβ (Moh y cols., 2011).

En células HeLa, la sobreexpresión de presenilinas PS1 y PS2 induce el arresto de las células en G_1 (Janicki y cols., 2000), mientras que su deficiencia acelera la transición de G_1 a S (Soriano y cols., 2001), lo que puede ser un nexo entre las mutaciones en *PS1* en alzhéimer familiar y las alteraciones del ciclo.

La excitotoxicidad también está relacionada con la activación del ciclo. Se ha observado que tras una lesión excitotóxica en el hipocampo, se expresan de manera secuencial marcadores del ciclo celular en la corteza entorrinal y el giro dentado (Hernandez-Ortega y cols., 2007). Ciclina D1 y Cdk6 (transición G_0/G_1) se expresan primero, seguidas de PCNA (G_1/S) que posteriormente disminuye para dejar paso a Cdk2 (S) y finalmente a ciclina B (G_2). Estos cambios secuenciales en respuesta a la excitotoxicidad son representativos de una reentrada en ciclo intencionada (Hernandez-Ortega y cols., 2007).

Las alteraciones en el ciclo celular también se relacionan con otros eventos patológicos de la enfermedad. Por ejemplo, el sistema ubiquitina-proteasoma media la proteólisis controlada de las ciclinas para permitir la progresión adecuada del ciclo, de modo que la alteración del sistema de ubiquitinación en EA puede influir en la regulación del ciclo celular en las neuronas (Tank y True, 2009).

En diversos estudios se ha visto que la patología neurofibrilar colocaliza con los reguladores del ciclo celular y que tau puede ser fosforilada por quinasas del ciclo, como Cdk2, Cdk5, Cdk1 y Cdc2-like (Baumann y cols., 1993; Bennecib y cols., 2000; Sobue y cols., 2000; Thakur y cols., 2008). La hiperfosforilación de tau puede causar problemas para la neurona cuando entra en ciclo, ya que la desorganización de la red de microtúbulos inicia un proceso denominado división prematura de los centrómeros, por el cual los centrómeros se dividen antes de tiempo, en la fase G_2 del ciclo, inmediatamente después de la replicación del ADN, lo que puede desencadenar la apoptosis. En la EA se ha encontrado que la incidencia de este proceso en la corteza es tres veces mayor que en controles

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(Spremo-Potparevic y cols., 2008).

El estrés oxidativo también está en estrecha relación con las alteraciones en el ciclo celular. El escaso número de neuronas que en un momento dado sufren apoptosis en una enfermedad crónica como la EA, llevó a Raina y cols. a sugerir el término abortosis, un proceso por el cual las neuronas activan las caspasas reguladoras, pero no las efectoras, de modo que pueden sobrevivir durante un tiempo al no completar la apoptosis (Raina y cols., 2001; Raina y cols., 2003). El estrés oxidativo crónico puede inducir la abortosis, como sugieren algunos estudios en los que la exposición a concentraciones bajas de oxidantes pueden dar lugar a una respuesta adaptativa en lugar de producir muerte neuronal (Crawford y Davies, 1994; Wiese y cols., 1995; Cassina y cols., 2001). El estrés oxidativo crónico al que están sometidas las neuronas en la EA puede provocar cambios compensatorios que permitan mantener la homeostasis, un estado en equilibrio en el que las neuronas sobreviven durante años (Zhu y cols., 2004b). Es en este estado de equilibrio cuando un "segundo impacto", la entrada en ciclo de esas neuronas, provocaría la muerte celular, según la teoría del doble impacto de Zhu y cols. (Zhu y cols., 2004b; Zhu y cols., 2007a). La situación también podría suceder al revés, pero sería necesaria la combinación de ambos factores para que tenga lugar la muerte neuronal.

Las alteraciones en el ciclo celular se encuentran también en pacientes de alzhéimer familiar (Malik y cols., 2008), en modelos animales (Yang y cols., 2006; Varvel y cols., 2009) y no sólo aparecen en neuronas, sino también en tejidos periféricos como fibroblastos (Tatebayashi y cols., 1995; Cavazzin y cols., 2004) y linfocitos (Stieler y cols., 2001; Nagy y cols., 2002; de las Cuevas y cols., 2003; Zhang y cols., 2003; Zhou y Jia, 2010; Stieler y cols., 2012). Asimismo, se han relacionado con otras enfermedades neurodegenerativas como el Parkinson, la demencia frontotemporal o la ataxia-telangectasia entre otras (Husseman y cols., 2000; Lee y cols., 2003; Yang y Herrup, 2005).

Por todos estos motivos, la EA se podría considerar un proceso neoplásico abortivo. Mientras que en el cáncer la entrada anormal en ciclo produce proliferación incontrolada y escape de la apoptosis, conduciendo al desarrollo y la malignización del tumor, en la EA la incapacidad para completar el ciclo celular desencadena la neurodegeneración.

5.1 Criterios NINCDS-ADRDA

En la actualidad, el diagnóstico de la EA se basa en los criterios del NINCDS-ADRDA *(National Institute of Neurologic, Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association)* (McKhann y cols., 1984). Según este protocolo, el diagnóstico definitivo de la enfermedad solamente se puede realizar con un análisis histopatológico post-mortem, y mientras tanto, se diagnostica como probable o posible EA. El diagnóstico clínico se realiza a través de los síntomas, la existencia de alteraciones cognitivas, sociales u ocupacionales y un análisis neuropsicológico. Entre las pruebas destaca el test mini-mental, MMSE (*Mini-Mental State Examination)* que explora distintas áreas cognitivas (orientación, fijación, concentración, cálculo, memoria y lenguaje) y otorga una puntuación en función de la cual los resultados pueden ser sugerentes de demencia (Roselli y cols., 2009). Desde que se publicaran estos criterios, se han descrito numerosas evidencias de que técnicas como la resonancia magnética, de imagen o de espectroscopía, la neuroimagen funcional o la búsqueda de marcadores en fluidos biológicos permiten caracterizar mejor la enfermedad.

5.2 Resonancia magnética

Esta técnica ha supuesto un avance tecnológico muy importante en las últimas décadas.

5 Diagnóstico

El fenómeno físico de la resonancia magnética nuclear sienta sus bases en el magnetismo. Los elementos con un número impar de electrones poseen una propiedad conocida como momento magnético o espín, que se caracteriza por presentar un campo magnético propio. Entre los átomos que presentan esta propiedad destaca el átomo de hidrógeno, que es el más abundante del cuerpo al formar parte de las moléculas de agua. En reposo, los campos magnéticos de cada átomo están orientados al azar. Sin embargo, cuando se somete la muestra a un campo magnético externo, semejante a un imán, los átomos se alinean con dicho campo magnético externo. Los campos magnéticos empleados en RM son de alta intensidad, medida en Teslas. La alineación es un proceso dinámico en el que cada átomo vibra con una frecuencia de giro característica. En el caso del átomo de hidrógeno, esta frecuencia es de 42.5 MHz/Tesla. Cuando se aplica una energía de la misma frecuencia que la del átomo de hidrógeno, es decir, cuando los átomos de hidrógeno y el campo magnético externo están en resonancia magnética, se produce un cambio en la orientación de los átomos. Cuando se interrumpe el campo magnético externo, los átomos vuelven a su posición original en un proceso de relajación por el que liberan la energía aplicada en forma de señal de radiofrecuencia, de diferente intensidad según el entorno químico en el que se encuentren (figura 18). Las señales se captan y se transforman de distintas formas. En el caso de la resonancia magnética de imagen, MRI (*Magnetic Resonance Imaging)* se transforman en funciones señal/tiempo, dando lugar a los tiempos de relajación T1/T2 para generar una imagen. En el caso de la resonancia magnética de espectroscopía (MRS, *Magnetic Resonance Spectroscopy)* las señales son ordenadas según su frecuencia para dar lugar a un espectro de frecuencias.

5.2.1 Resonancia magnética de imagen (MRI)

Las imágenes obtenidas pueden ser de distintos tipos.

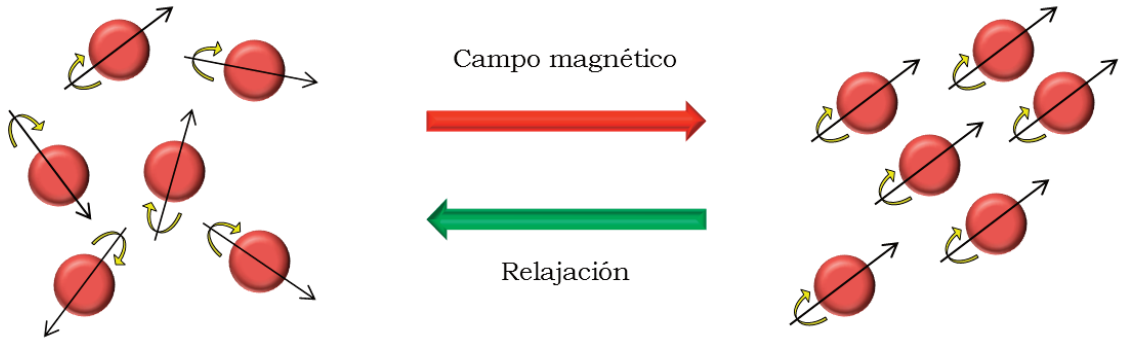

Figura 18. Esquema de la obtención de una señal de resonancia magnética.

❍ Imágenes pesadas en T1 y T2

Las señales de radiofrecuencia generadas se captan en funciones de señal-tiempo y se convierten en una escala de grises para formar una imagen. En ellas, se clasifica la señal como brillante o hipertensa cuando aparece blanca, o como hipotensa cuando aparece oscura. El proceso de relajación de los átomos de hidrógeno hasta su posición original se puede dividir en dos partes (imposibles de distinguir en la práctica) que corresponden a los componentes vectoriales del eje de orientación de los átomos en cuestión. El componente vertical o longitudinal, depende de la interacción de los átomos con su entorno y, si se representa en función del tiempo, da lugar a una curva exponencial ascendente que se conoce como tiempo de relajación longitudinal o T1. El componente transversal depende de la interacción de los átomos entre sí, y su comportamiento en el tiempo es una curva exponencial descendiente que se corresponde con el tiempo de relajación transversal o T2. Las imágenes pesadas en T1 se caracterizan porque en ellas el líquido aparece oscuro, como una señal hipotensa. En el caso de las imágenes pesadas en T2, la materia grasa se muestra oscura y el agua brillante o hipertensa. Por lo tanto, debido a que en general el agua libre se asocia con la patología, esta técnica es muy útil en el estudio de diversas enfermedades cerebrales. Las imágenes obtenidas de esta manera permiten analizar las estructuras cerebrales y realizar estudios volumétricos.

❍ Mapas de Coeficiente de Difusión Aparente

Además, dentro de la imagen por resonancia, existen variantes cuantitativas, como la difusión en resonancia magnética, que permite detectar el grado de movimiento de las moléculas de agua en el medio extracelular, condicionado por la cantidad de células o la integridad de las membranas. El acúmulo regional de agua en el cerebro se puede producir por edema, inflamación, desmielinización u otras causas. Esta técnica mide la autodifusión, que es el movimiento de agua entre otras moléculas de agua. Es importante destacar que en los tejidos biológicos no existe una difusión libre, ya que las membranas se presentan como obstáculos a la difusión, de modo que la difusión del agua en los tejidos se mide como difusión aparente. La señal obtenida puede ser medida por medio del coeficiente de difusión aparente (ADC, *Apparent Diffussion Coeficient).* Cuando éste es calculado para cada píxel de la imagen se obtiene un mapa paramétrico, mapa de ADC, en el cual las áreas que no permiten la difusión aparecen en tonos azules, mientras que las que tienen difusión libre aparecen en tonos rojos (Vallejo Desviat y cols., 2011).

❍ Mapas de Transferencia de Magnetización

Por otro lado, la transferencia de magnetización (MT, *Magnetization Transfer),* se basa en

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el intercambio de magnetización entre los protones libres (representados por el agua) y los inmóviles, es decir, ligados a macromoléculas. Las biomoléculas que más agua acoplan son los fosfolípidos. El agua acoplada a biomoléculas se considera "invisible" ya que no transfiere magnetización a otras moléculas, mientras que el agua libre sí lo hace. La transferencia de magnetización entre ellos se produce al aplicar un pulso de presaturación (pulso MT) que hace que los protones del agua ligada intercambien su magnetización con la población de protones del agua libre, haciendo que la intensidad de la señal del agua libre descienda. El porcentaje de transferencia de magnetización (% MT) se usa como parámetro de medida del fenómeno. Las medidas de % MT dependen del entorno químico y biofísico que rodea las macromoléculas y sirven por lo tanto para medir indirectamente la estructura macromolecular del tejido en cuestión, indicando posibles cambios estructurales, por ejemplo, en el parénquima cerebral (Grossman y cols., 1994).

5.2.2 Resonancia Magnética de Espectroscopía (MRS)

La espectroscopía de resonancia magnética se diferencia de la de imagen en la manera de analizar las señales. En lugar de una imagen anatómica, se obtiene un espectro de componentes bioquímicos correspondientes a los principales metabolitos cerebrales. El *hardware* empleado es el mismo que para la obtención de imágenes de RM, de manera que es una técnica ampliamente utilizada. Los espectros se representan como gráficos compuestos por múltiples picos en los que el área bajo cada pico constituye la concentración relativa de una especie química dada. Los metabolitos se diferencian por su posición en el espectro, ya que su diferente composición química hace que su resonancia sea también diferente. Entre los metabolitos que permite detectar esta técnica en un volumen cerebral concreto destacan el N-acetil-

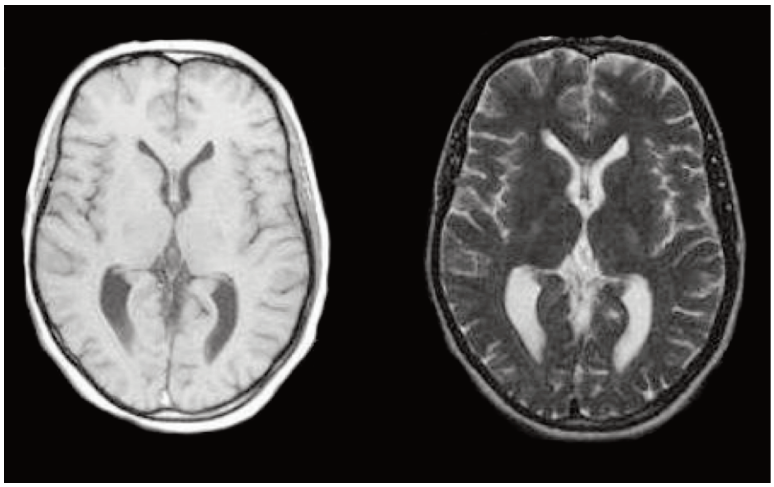

Figura 19. Ejemplo de imágenes de resonancia magnética pesadas en T1 (izquierda) y en T2 (derecha). Imagen tomada de la página web de la Asociación Holandesa de Neurocirugía http://www.nvvn.org/.

aspartato (NAA), cuya disminución se considera un marcador de muerte neuronal; el mioInositol (mI), característico de las células gliales; la colina (Cho), marcador de la integridad y la síntesis de membranas, que indica la concentración de precursores de membrana como colina y fosfocolina; o la creatina (Cr), que suele ser el pico más estable y se considera como control de los demás metabolitos (Alvarez-Linera Prado y cols., 2003). Un ejemplo de un espectro se puede observar en la figura 20.

5.2.3 MRI Y MRS en la EA

En la EA, la MRI se emplea en primer lugar para detectar posibles lesiones intracraneales como tumores o hematomas, que puedan contribuir a la demencia (Waldemar y cols., 2007). Además, se usa para medir la atrofia del lóbulo temporal medial, característica que permite diferenciar con una gran sensibilidad y especificidad los pacientes con EA de los controles, y en algunos casos identificar pacientes con DCL que pueden desarrollar la enfermedad (Chincarini y cols., 2011; Westman y cols., 2011). Esta técnica también es útil para medir el volumen cerebral y de estructuras cerebrales específicas. En ese sentido, la atrofia del hipocampo y de la corteza entorrinal y el ensanchamiento de los ventrículos son características de los pacientes de EA que se pueden medir por MRI (Fleisher y cols., 2008; Nestor y cols., 2008). En cuanto a los metabolitos cuantificados empleando espectroscopía de resonancia magnética, el hallazgo más consistente es la reducción del pico de N-acetil-aspartato observado en distintas regiones del cerebro, que puede dar idea de

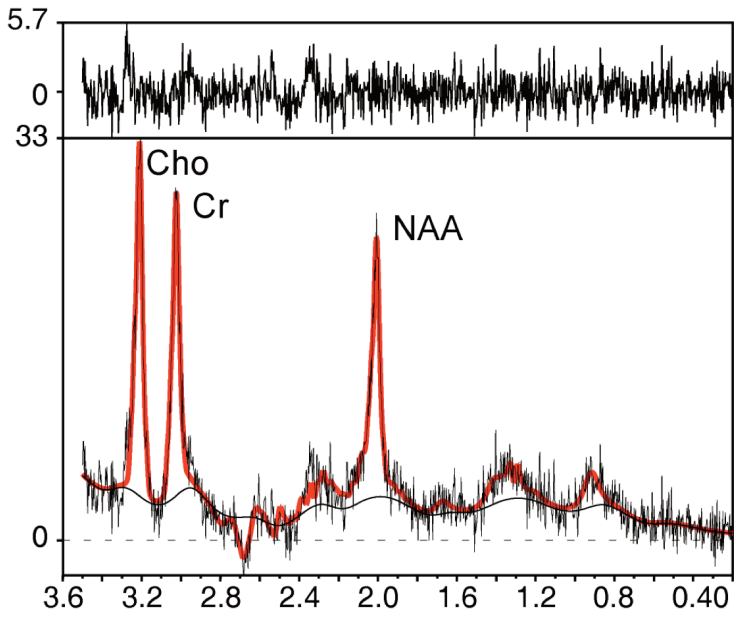

Figura 20. Ejemplo de un espectro obtenido por MRS. Se pueden distinguir tres metabolitos característicos: NAA, Cr y Cho.

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la muerte neuronal (Kantarci, 2007). En relación a las técnicas cuantitativas, existen evidencias de que el coeficiente de difusión aparente se incrementa en la enfermedad (Back y cols., 2003; Clerx y cols., 2012), mientras que la transferencia de magnetización es menor (Ginestroni y cols., 2009; Kiefer y cols., 2009).

5.2.4 Técnicas de neuroimagen funcional

Las técnicas de neuroimagen funcional también son muy interesantes desde el punto de vista diagnóstico. Destaca la tomografía por emisión de positrones, PET *(Positron Emission Tomography)*, que es capaz de medir la actividad metabólica de los tejidos tras detectar la distribución que adopta en ellos un radiofármaco de vida media ultracorta tras ser administrado por vía intravenosa. En el caso de la EA, la administración de fluorodeoxiglucosa permite medir el metabolismo de glucosa en el cerebro y distinguir individuos control de pacientes, ya que éstos últimos presentan menor consumo de glucosa en la región temporal-parietal (Mosconi, 2005; Jagust y cols., 2007). Otro ligando que puede emplear el PET es el ^{11}C-PiB, que se usa para visualizar Aβ in vivo. Una mayor retención de este ligando se relaciona con la EA (Forsberg y cols., 2010).

5.3 Marcadores en fluídos biológicos

Por último, la búsqueda de marcadores en fluidos biológicos también es de gran interés. El líquido cefalorraquídeo, caracterizado por su composición constante y su estrecha relación con el sistema nervioso, ha centrado una buena parte de la búsqueda de marcadores diagnósticos. Los resultados destacan que la disminución en la concentración de Aβ42 y el incremento de tau total o fosforilada en este fluido permiten diferenciar a los pacientes de los controles (Mattsson y cols., 2009; Tapiola y cols., 2009; Seppala y cols., 2012). También se han encontrado potenciales marcadores periféricos en fluidos más fácilmente accesibles como el plasma, en el que además de Aβ se han encontrado otros marcadores, entre ellos algunos relacionados con estrés oxidativo e inflamación (Kim y cols., 2011; Song y cols., 2011; Torres y cols., 2011). La medición de los niveles de Aβ42 en saliva también puede considerarse un biomarcador periférico potencial (Bermejo-Pareja y cols., 2010).

Por estos motivos, algunos autores piden una revisión de los criterios del NINCDS-ADRDA para que, además del diagnóstico clínico, sea necesaria la presencia al menos, de uno de los marcadores mencionados con anterioridad (cambios volumétricos medidos con MRI, alteraciones metabólicas medidas con PET o marcadores en el líquido cefalorraquídeo) (Dubois y cols., 2007). En los últimos años, la iniciativa ADNI *(Alzheimer's Disease Neuroimaging Initiative)* está realizando un

estudio longitudinal multicéntrico a nivel mundial para la búsqueda de marcadores para el diagnóstico precoz y seguimiento de la EA (Weiner y cols., 2012).

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El hallazgo de un tratamiento eficaz que detenga o ralentice la progresión de la EA sigue siendo uno de los objetivos más importantes de la investigación contra esta enfermedad. En la actualidad, solamente existen cinco fármacos aprobados por la FDA (*Food and Drug Administration*) y la Agencia Europea del Medicamento (EMA, *European Medicines Agency)* para el tratamiento de la EA, aunque están en marcha numerosos ensayos clínicos con fármacos que actúan en distintos mecanismos implicados en su patogénesis.

6.1 Tratamientos aprobados en la actualidad

❍ *Inhibidores de la acetilcolinesterasa*

El objetivo de estos fármacos es combatir el déficit colinérgico impidiendo la degradación de la acetilcolina en el espacio sináptico. La tacrina fue el primer inhibidor de la acetilcolinestarasa (AchEI) aprobado por la FDA en 1993, sin embargo, está en desuso por su hepatotoxicidad (Watkins y cols., 1994). Posteriormente se aprobaron otros tres AchEI, cada uno de ellos con un mecanismo de acción diferente: donepezilo (1997), el más prescrito en la actualidad; rivastigmina (2000) y galantamina (2001) (Fan y Chiu, 2010). En general, el análisis de numerosas revisiones de la última década señala que estos fármacos tienen un efecto positivo en la cognición, el comportamiento y las actividades de la vida diaria en pacientes con EA leve a moderada, mejorando

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su calidad de vida y la de sus cuidadores. En alguno de estos pacientes incluso se encontró una mejora en las funciones cognitivas tras 1 o 2 años de terapia, revisado en (Martorana y cols., 2010). En general, muchos estudios apuntan que estos tratamientos son altamente coste-efectivos (Getsios y cols., 2010; Hartz y cols., 2012), aunque el coste-efectividad a largo plazo es un tema controvertido (Keller y cols., 2011; Versijpt, 2012).

❍ *Antagonistas de los receptores NMDA*

El último de los fármacos aprobados hasta el momento es la memantina. Es un antagonista de los receptores ionotrópicos NMDA de glutamato, que actúa impidiendo la sobreestimulación de los mismos y protegiendo a las neuronas de la excitotoxicidad mediada por calcio (Lipton, 2005; Blanchard y cols., 2008).

El fármaco se aprobó en 2003 para el tratamiento de la EA moderada y severa, tanto en monoterapia como en combinación con donepezilo (Emre y cols., 2008). Numerosos estudios han mostrado su eficacia en la mejora de las funciones cognitivas y de comportamiento (Thomas y Grossberg, 2009; Lo y Grossberg, 2011). Otras revisiones también consideran el fármaco coste-efectivo (Antonanzas y cols., 2006; Rive y cols., 2010).

6.2 Estrategias terapéuticas en estudio

6.2.1 Estrategias para prevenir la producción de Aβ

❍ *Inhibidores de la β-secretasa*

La β-secretasa (BACE1) es la primera proteasa que participa en la vía amiloidogénica, de modo que su inhibición podría prevenir la formación de Aβ y favorecer la vía no amiloidogénica. La inhibición directa de la enzima es problématica, debido a la gran cantidad de sustratos fisiológicos que tiene, incluído uno implicado en la mielinización (Klaver y cols., 2010). De momento, sólo un fármaco, CTS-21166, ha llegado a la fase I de los ensayos clínicos, revisado en (Ghosh y cols., 2012).

La modulación indirecta de la β-secretasa podría ser una alternativa, y es posible a través de mecanismos que incluyen al receptor nuclear PPARγ *(Peroxisome Proliferator-Activated Receptor)*. Cuando éste se estimula, se reduce la expresión de BACE1 y por lo tanto de APP (d'Abramo y cols., 2005; Sastre y cols., 2006a). Debido al importante papel de PPARγ en el metabolismo de lípidos y glucosa, es una importante diana en el tratamiento de la diabetes tipo II, y en ese sentido se han desarrollado fármacos como la rosiglitazona. Experimentos con este fármaco en ratones muestran efectos beneficiosos en EA (Escribano y cols., 2010), pero no se ha demostrado la misma eficacia en ensayos clínicos en los que se emplea este fármaco sólo, o en combinación con AchEI (Gold y cols., 2010; Harrington y cols., 2011).

❍ *Inhibidores de la γ-secretasa*

En este caso, el objetivo es impedir la formación de β-amiloide inhibiendo la acción de la última enzima encargada de su procesamiento, la γ-secretasa. Esta enzima también es parte esencial de la ruta de señalización de Notch, implicada en procesos de diferenciación celular (Kopan y Ilagan, 2009), por lo que el principal obstáculo es encontrar inhibidores de γ-secretasa que no interfieran con Notch para evitar los efectos tóxicos que supone su inhibición (Searfoss y cols., 2003; Wong y cols., 2004). El ensayo clínico más reciente con un inhibidor de la γ-secretasa (semagacestat) completó con éxito la fase II, pero se detuvo en la fase III cuando se observó un empeoramiento de los pacientes, al parecer por toxicidad en las sinapsis (http://www.alzforum.org/).

Algunas drogas, en concreto algunos antiinflamatorios no esteroideos, son capaces de modular la γ-secretasa y disminuir la producción de Aβ42 favoreciendo la producción de formas más cortas (Eriksen y cols., 2003). El primero de este tipo de fármacos en ensayo clínico fue tarenflurbil (R-flurbiprofeno), aunque no mostró efectos beneficiosos en fase III (Green y cols., 2009). En la actualidad, una amplia gama de este tipo de compuestos están en estudio, revisado en (Wolfe, 2012).

❍ *Inmunoterapia*

Inmunoterapia activa

La inmunoterapia activa consiste en la administración del péptido Aβ sintético o un fragmento del mismo para inducir la producción de anticuerpos anti-Aβ que eliminen Aβ del cerebro. La primera aproximación exitosa a este tipo de terapias se produjo en 1999, cuando la inmunización con Aβ de un ratón transgénico PDAPP produjo una disminución de las placas y de la distrofia de las neuritas (Schenk y cols., 1999). En humanos, el primer ensayo clínico con este tipo de vacunas (AN1792) mostró efectos adversos como meningoencefalitis en un 6% de los pacientes, por lo que fue abandonado (Senior, 2002). Desde entonces, la segunda generación de vacunas intenta evitar estos efectos adversos y ha mostrado disminuir las placas y el amiloide insoluble en el cerebro, pero no el soluble o los oligómeros (Kim y cols., 2004).

Inmunoterapia pasiva

Consiste en la administración de un anticuerpo monoclonal que se une a Aβ y permite aumentar su eliminación del cerebro. En general, los estudios en ratones transgénicos con este tipo de terapia muestran una disminución en el número de placas y una mejora en el déficit cognitivo (Bard y cols., 2000; Wilcock y cols., 2004). En humanos, la inmunoterapia pasiva con bapineuzumab, un anticuerpo monoclonal humanizado contra Aβ, ha

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mostrado en fase II reducir la carga de Aβ en el cerebro, aunque algunos pacientes han desarrollado edemas vasculares y microhemorragias. De momento, aún no ha mostrado beneficios cognitivos (Panza y cols., 2011; Panza y cols., 2012). Solanezumab es otro anticuerpo monoclonal que actualmente se encuentra en fase III. Los beneficios cognitivos que mostró en ratones, su capacidad para neutralizar selectivamente especies solubles de Aβ y la ausencia de efectos adversos en fase II, hacen de esta vacuna un fármaco prometedor (Samadi y Sultzer, 2011; Imbimbo y cols., 2012). En la actualidad, existen más de diez vacunas en diversas fases de los ensayos clínicos, revisado en (Delrieu y cols., 2012).

6.2.2 Otras estrategias

Además de los antiinflamatorios mencionados en el capítulo correspondiente, las siguientes dianas también son potenciales estrategias terapéuticas contra la EA.

❍ *Tau*

Se han diseñado estrategias dirigidas contra las quinasas responsables de la fosforilación de tau, entre ellas GSK3β y Cdk5 (Lopez-Tobon y cols., 2011). Aunque algunos compuestos como el ácido valproico (aintiepiléptico) y el litio mostraron inhibir la GSK3, los ensayos se abandonaron por los efectos colaterales en un caso y por no reducir la fosforilación de tau en el segundo caso (Hampel y cols., 2009; Tariot y Aisen, 2009). En la actualidad está en fase III el methylen blue (Fan y Chiu, 2010), un compuesto que en estudios preclínicos mostró una mejora de los niveles cognitivos y una reducción de los niveles de Aβ y tau (Medina y cols., 2011; Congdon y cols., 2012).

Las vacunas contra tau han mostrado resultados satisfactorios en ratones (Chai y cols., 2011).

❍ *Antioxidantes*

Algunos estudios epidemiológicos han mostrado que el consumo de antioxidantes como vitamina C o E, carotenos, flavonoides o polifenoles en la dieta se asocia con un menor riesgo de desarrollar alzhéimer (Devore y cols., 2010; Craggs y Kalaria, 2011).

Los primeros estudios con extractos de Ginkgo Biloba, un conocido antioxidante, mostraron efectos positivos sobre la cognición, el aprendizaje y la memoria, sin embargo, ensayos clínicos recientes no han mostrado diferencias entre Ginkgo Biloba y placebo (Birks y cols., 2002; Snitz y cols., 2009). La curcumina, una especia del curry, ha mostrado en estudios con animales disminuir la patología de Aβ y el estrés oxidativo (Lim y cols., 2001). Sin embargo, los resultados de los ensayos clínicos no avalan estas observaciones (Hamaguchi y cols., 2010). Lo mismo sucede con el ácido

docosahexaenoico, un ácido graso poliinsaturado omega 3. Los estudios en animales mostraron reducir la inflamación, el estrés oxidativo y la patología Aβ (Lim y cols., 2005), sin embargo, los ensayos clínicos no han mostrado esos beneficios (Jicha y Markesbery, 2010). El consumo moderado de vino tinto, también se asoció con un menor riesgo de alzhéimer. Este efecto podría estar mediado por el resveratrol, un polifenol que ha mostrado efectos neuroprotectores en modelos animales y celulares (Vingtdeux y cols., 2008; Capiralla y cols., 2012).

❍ *Calcio*

Además de la memantina, antagonista de los receptores NMDA, existen numerosos potenciales tratamientos que se dirigen al resto de canales de calcio, revisado en (Yu y cols., 2009a). Entre otros, se ha estudiado la eficacia de los bloqueantes de canales de calcio dependientes de voltaje, que en la actualidad se emplean para el tratamiento de la hipertensión. El nimodipino parece mejorar la función cognitiva en algunos estudios, aunque hasta la fecha no hay ensayos clínicos exhaustivos (Lopez-Arrieta y Birks, 2002). El isradipino es otro bloqueante que *in vitro* ha mostrado buenos resultados (Anekonda y Quinn, 2011). Dantrolene, un antagonista del receptor de rianodina, mejora la cognición en un modelo murino de EA (Peng y cols., 2012). Otros fármacos como los antiinflamatorios indometacina, ibuprofeno o R-flurbiprofeno protegen a las neuronas de la toxicidad por Aβ inhibiendo la sobrecarga de calcio en la mitocondria (Sanz-Blasco y cols., 2008).

❍ *Estatinas*

A pesar de los buenos resultados de los estudios epidemiológicos, los ensayos clínicos con estatinas muestran resultados contradictorios (Shepardson y cols., 2011b; Shepardson y cols., 2011a). Aunque algunos ensayos clínicos han mostrado beneficios en las funciones cognitivas tras el tratamiento con simvastatina o atorvastatina, (Sparks y cols., 2005; Carlsson y cols., 2008) otros ensayos recientes no lo hacen (Feldman y cols., 2010; Sano y cols., 2011).

❍ *Ciclo celular*

Los objetivos de este tipo de tratamiento serían evitar la transición G_1/S o la replicación del ADN. En el primer caso se engloban los inhibidores de Cdks. Muchos de ellos ya existen para el tratamiento contra el cáncer. Hasta el momento, los ensayos con flavopiridol, olomucina y roscovitina han mostrado ser neuroprotectores en estudios *in vitro*, aunque ninguno de ellos se ha probado en modelos animales (Verdaguer y cols., 2003; Pallas y cols., 2005; Alvira y cols., 2007; Hilton y cols., 2008; de la Torre y cols., 2012). En el segundo caso, los inhibidores de la ADN polimerasa b como el ácido litocólico o la dedioxicitidina podrían ser una diana potencial específica de la inhibición del ciclo

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neuronal, aunque son necesarios más estudios que confirmen esta hipótesis (Copani y cols., 2008). Por otro lado, existen numerosos compuestos farmacológicos potencialmente útiles en el alzhéimer y que indirectamente pueden modular el ciclo celular. Por ejemplo, algunos componentes de la dieta como los ya mencionados curcumina o resveratrol, pueden ejercer sus efectos beneficiosos controlando el ciclo celular a través de la regulación de las Cdks (Meeran y Katiyar, 2008). Entre otros extractos naturales destaca la epigallocatethina-gallato, un componente del té verde que ha mostrado actuar en la fase G_0/G_1 del ciclo (Snape y cols., 2009). En la actualidad, se encuentra listado como inhibidor del ciclo celular en un ensayo clínico en fase III contra el alzhéimer http://clinicaltrials.gov/ct2/show/NCT00951834.

La rosiglitazona y la pioglitazona, agonistas de PPARγ que modulan indirectamente la β-secretasa, también causan arresto en G_0/G_1 (Heaney y cols., 2003), al igual que algunas estatinas como la simvastatina (Chan y cols., 2008; Wali y cols., 2009; Relja y cols., 2010). Hay que señalar que las estatinas, al inhibir la ruta del mevalonato, afectan a la isoprenilación de proteínas como Ras, que puede ser una inductora del ciclo celular en neuronas (Chang y cols., 2003). Los antinflamatorios no esteroideos también han sido capaces de prevenir las alteraciones del ciclo celular en un modelo de ratón (Varvel y cols., 2009). Otros compuestos que se han mostrado neuroprotectores en modelos animales y que producen arresto en G_0/G_1 son la vitamina A (ácido retinoico), la vitamina D (calcitriol), los glucocorticoides o la taurina, entre otros, revisado en (Woods y cols., 2007).

❍ *Estimulación de la neurogénesis y tratamiento con células madre*

La neurogénesis es el proceso que comprende la proliferación, migración y diferenciación de las células progenitoras neuronales en un tipo específico de neurona y su integración en el circuito neuronal adulto (Laplagne y cols., 2006). Las poblaciones de células progenitoras neurales están presentes en nichos especializados del cerebro, como la zona subventricular, el bulbo olfatorio y el giro dentado del hipocampo (Mu y Gage, 2011). El proceso de neurogénesis disminuye con la edad, y con algunos estímulos como el estrés o la depresión, y se incrementa con la presencia de factores de crecimiento como NGF o BDNF (*Brain-Derived Neurotrophic Factor)*, y con el tratamiento con antidepresivos o estrógenos, entre otros, revisado en (Abdel-Salam, 2011). Existe controversia respecto a la neurogénesis en EA; mientras que algunos autores describen una disminución de la neurogénesis en pacientes y modelos animales, y lo consideran un evento temprano de la enfermedad (Ziabreva y cols., 2006; Mu y Gage, 2011), otros autores encuentran una elevada neurogénesis que se interpreta como un intento de establecer un mecanismo de compensación contra la pérdida neuronal (Jin y cols., 2004; Chen y cols.,

2008; Yu y cols., 2009b).

El estímulo de la neurogénesis podría ser, por lo tanto, una estrategia atractiva para movilizar los precursores neuronales y hacer que produzcan neuronas que permitan sustituir a las dañadas, principalmente en el hipocampo. La galantamina, tacrina y memantina aprobadas para el uso de la EA, estimulan la neurogénesis en modelos de ratón (Jin y cols., 2006), un proceso que puede estar mediado por BDNF (Leyhe y cols., 2008). Otras drogas en estudio in vitro son FK-960, SGS-111, piracetam o quercetina revisado en (Abdel-Salam, 2011).

El transplante de células madre humanas establecidas también se está estudiando en modelos murinos. Algunos modelos (Yamasaki y cols., 2007; Xuan y cols., 2009) han mostrado que estas células transplantadas pueden sobrevivir, migrar y diferenciarse a neuronas colinérgicas, mostrando una mejora de las funciones cognitivas. En otros casos, la implantación de células madre puede actuar por otros mecanismos, como incrementar la proliferación de los progenitores neuronales endógenos (Mahmood y cols., 2004; Munoz y cols., 2005), modular la neuroinflamación (Taupin, 2009) o estimular la producción de factores neurotróficos como NGF o BDNF (Luo y cols., 2009). En cualquier caso, aunque se necesitan más estudios para ver los efectos a largo plazo de este tipo de técnicas, como la posibilidad de formación de tumores, pueden ser una estrategia interesante contra la pérdida neuronal.

7 Modelos de estudio de la enfermedad

Muchos de los estudios que se han realizado hasta la actualidad sobre la EA emplean material de autopsia de pacientes. Además de los problemas vinculados al uso de tejidos post-mortem, este tipo de muestras están en general asociadas a la patología terminal y no permiten estudiar la progresión de la enfermedad. En algunas ocasiones también se puede emplear tejido nervioso de pacientes vivos obtenido por cirugía, pero la difícil accesibilidad complica este tipo de experimentos. Otros modelos alternativos para el estudio de la enfermedad a nivel celular y molecular son los animales y las células periféricas de pacientes.

7.1 Modelos animales

Durante los últimos veinte años, se han desarrollado un gran número de ratones transgénicos que sobreexpresan APP y/o presenilina, principalmente PS1, basándose en las mutaciones encontradas en los casos de alzhéimer familiar. Los ratones transgénicos desarrollan progresivamente muchas de las principales características de EA, como elevados niveles de Aβ, depósitos de amiloide, neuroinflamación, estrés oxidativo, gliosis, pérdida sináptica y déficit cognitivo; sin embargo, ni los ovillos neurofibrilares ni la muerte neuronal son patentes en la gran mayoría de los modelos (Balducci y Forloni, 2011).

7 Modelos de estudio de la enfermedad

Para generar las líneas transgénicas se emplean las tres isoformas de APP humana (695, 751 y 770 hAPP), y se introducen mutaciones encontradas en la enfermedad familiar, nombradas según el área geográfica en la que se descubrieron. Destacan la doble mutación *Swedish* (K670N, M671L) que aumenta la producción de Aβ; la mutación *London* (V717I) e *Indiana* (V717F) que aumentan preferentemente la forma Aβ42; la mutación *Arctic* (E693G) que disminuye el procesamiento por la α-secretasa; y la mutación *Dutch* (E693Q) que aumenta el ratio Aβ40/Aβ42, revisado en (Chin, 2011). Los diferentes promotores empleados permiten la expresión preferencial o exclusiva de hAPP en el SNC. Una manera de acelerar la deposición de placas es la co-transfección de *APP* y *PS1* mutados. Las mutaciones en *PS1* son de distintos tipos, destacando PS1(A246E), PS1(M146L) y PS1(M146V), y se combinan con las mutaciones en *APP* para generar diferentes tipos de modelos doble transgénicos. Un resumen de las características de estos ratones se encuentra en la tabla 1. A pesar de que presentan tau hiperfosforilada, estos modelos no desarrollan ovillos neurofibrilares, de modo que para investigar su papel, se escogen modelos basados en mutaciones en *tau*, entre los que destaca el triple transgénico (3xTg) con mutaciones en *APP, PS1* y *tau* (Oddo y cols., 2003). Otro de los aspectos claves de EA, la muerte neuronal, tampoco es evidente en la gran mayoría de modelos murinos, aunque sí se ha observado en algunos, como en el APP23 cuando los ratones alcanzan 14 meses de edad (Calhoun y cols., 1998). Otros modelos más "agresivos", ratones que combinan dos o tres mutaciones en *APP* y dos en *PS1*, como el 5FAD, han mostrado pérdida neuronal en el hipocampo en etapas más tempranas (Casas y cols., 2004; Oakley y cols., 2006).

Aunque ninguno de los modelos reproduce totalmente las características de la enfermedad en humanos (algunos autores consideran que la vida del ratón es demasiado corta para poder hacerlo) estos modelos permiten estudiar muchos de los aspectos relacionados con la EA, así como valorar posibles tratamientos terapéuticos. El establecimiento de líneas celulares neuronales o extraneuronales, o los cultivos primarios de neuronas y astrocitos a partir de estos ratones son técnicas muy útiles para el estudio de la enfermedad.

Modelo	Mutación	Promotor	Placas de amiloide	Pérdida Neuronal	Ovillos	Déficit cognitivo	Referencia
PDAPP	APP 695, 751, 770 (APPInd)	PDGF-β	6-9 meses	No	No	Sí	(Games y cols., 1995)
Tg2576	APP695 (APPSwe)	Hamster PrP	9 meses	No	No	Sí	(Hsiao y cols., 1996)
APP23	APP751 (APPSwe)	Mouse Thy-1	6 meses	14 meses	No	Sí	(Calhoun y cols., 1998)
J20	APP 695, 751, 770 (APPSwe, Ind)	PDGF-β	6 meses	No	No	Sí	(Mucke y cols., 2000)
TgCRND8	APP 695 (APPSwe, Ind)	Hamster PrP	3 meses	No	No	Sí	(Chishti y cols., 2001)
APP/PS1	Tg2576 X PS1 (M146L)	Hamster PrP PDGF-β	6 meses	No	No	Sí	(Holcomb y cols., 1998)
5xFAD	APP695 (Swe, Lon, Flo) PS1 (M146L, L286V)	Mouse Thy-1	2 meses	9 meses	No	Sí	(Oakley y cols., 2006)
rTg4510	4RoN MAPT (Tau-P301L)	Mouse PrP	No	6 meses	4 meses	Sí	(Santacruz y cols., 2005)
3xTG	APP695(Swe) Tau (P301L) PS1 (M146V)	Mouse Thy-1.2	6 meses	No	12 meses	Sí	(Oddo y cols., 2003)

Tabla 1. Diferentes modelos de ratones transgénicos empleados en el estudio de la EA. Adaptado de (Chin, 2011).

7 Modelos de estudio de la enfermedad

7.2 Tejidos periféricos de pacientes

El empleo de tejidos periféricos de pacientes de alzhéimer es una estrategia alternativa y complementaria que tiene múltiples ventajas, entre ellas, la fácil accesibilidad de las células y la posibilidad de obtenerlas en cualquier momento del curso de la enfermedad. Los principales tejidos periféricos que se emplean para el estudio de la EA son células sanguíneas, como linfocitos o plaquetas, y fibroblastos (Etcheberrigaray y Ibarreta, 2001; Casoli y cols., 2008). El uso de este tipo de células se basa en estudios que hacen pensar que la EA también tiene manifestaciones sistémicas, aunque con menores repercusiones clínicas que en el cerebro (Blass y Gibson, 1992; Scott, 1993; Gasparini y cols., 1998).

Las células periféricas son útiles para comprender los mecanismos implicados en la patogénesis de la enfermedad ya que muchas de las alteraciones encontradas en los cerebros de los pacientes y en modelos transgénicos aparecen también en células periféricas. En linfocitos, por ejemplo, se han mostrado alteraciones en el ciclo celular (Urcelay y cols., 2001; Zivkovic y cols., 2010; Stieler y cols., 2012), estrés oxidativo y alteraciones mitocondriales (Gibson y Huang, 2002; Leuner y cols., 2007; Sultana y cols., 2011), en la homeostasis del calcio (Eckert y cols., 1997; Ibarreta y cols., 1997; Nelson y cols., 2010) o alteraciones en genes relacionados con la producción de Aβ y el procesamiento de tau (Maes y cols., 2007). Además, existe una estrecha relación entre el estado del sistema inmune (principalmente los linfocitos) y las enfermedades neurodegenerativas (Schwarz y cols., 2001; Pellicano y cols., 2012). Por estos motivos, algunos autores afirman que existe una comunicación bidireccional entre el sistema nervioso central y el inmune a través de un complejo mecanismo que engloba hormonas, citoquinas y neurotransmisores (Gladkevich y cols., 2004). Por ejemplo, existen receptores de neurotransmisores y factores de crecimiento como BDNF o NGF, entre muchos otros, que se expresan tanto en neuronas como en linfocitos, revisado en (Gladkevich y cols., 2004). En cualquier caso, es necesario evaluar adecuadamente los resultados obtenidos en estos tipos celulares, ya que los fenómenos observados en estos tejidos no son siempre necesariamente extrapolables al sistema nervioso.

Los fibroblastos, por su parte, también muestran alteraciones en la homeostasis del calcio (Palotas y cols., 2002), estrés oxidativo y alteraciones mitocondriales (Wang y cols., 2008; Mendonsa y cols., 2009), secreción de Aβ en fibroblastos de pacientes de EA familiar (Johnston y cols., 1994) y alteraciones en el ciclo celular (Tatebayashi y cols., 1995). Además, son la base de un nuevo modelo para el estudio de la enfermedad, como son las células madre con pluripotencialidad inducida (iPSC, *Induced Pluripotent Stem Cells*).

Estas células se consiguen reprogramando los fibroblastos de pacientes hasta un estado indiferenciado por medio de la transfección mediada por retrovirus de distintos factores de transcripción. Posteriormente, las iPSC son diferenciadas a neuronas y, en el caso de la EA, han mostrado reproducir las características de la enfermedad (Yagi y cols., 2011; Israel y cols., 2012). También es posible obtener iPSCs a partir de linfocitos inmortalizados con el virus de Esptein-Barr (Choi y cols., 2011; Rajesh y cols., 2011). Aunque se ha conseguido diferenciar estas células a tejido neuronal, de momento no existen estudios sobre la EA en iPSCs obtenidas de células sanguíneas (Rajesh y cols., 2011).

El principal problema de la obtención de iPSCs es que es un proceso largo que se asocia con tumorogénesis e inestabilidad del ADN. Por ello, se está trabajando en la conversión directa de fibroblastos a células neuronales sin necesidad de pasar primero por el estado pluripotente. Qiang y cols. han logrado generar las llamadas hiN (*Human-Induced Neuronal Cells)* a partir de fibroblastos de pacientes de EA portadores de una mutación en presenilina, mediante co-transducción viral de distintos factores de transcripción en presencia de factores de crecimiento neuronales. Las hiN obtenidas muestran el perfil de las neuronas glutaminérgicas de la corteza y un incremento en el cociente Aβ42/40 en consonancia con la patología de la EA (Qiang y cols., 2011).

En conjunto, estos trabajos ponen de manifiesto el gran potencial de la reprogramación celular para obtener modelos neuronales para el estudio de la EA, así como para evaluar la eficacia de los tratamientos farmacológicos.

Objetivos

2

Objetivos

El objetivo general de esta tesis ha sido profundizar en el estudio de los mecanismos que regulan los procesos de proliferación/supervivencia/muerte celular en la enfermedad de Alzheimer. La muerte neuronal es una de las características más importantes de la EA, sin que hasta el momento se conozcan por completo las causas que producen la pérdida selectiva de neuronas en zonas específicas del cerebro. En una enfermedad tan compleja como la EA, los síntomas y el diagnóstico se producen cuando ya existe una pérdida neuronal significativa. Sin embargo, existen evidencias que indican que las características neuropatológicas de la enfermedad comienzan a aparecer mucho antes en la vida del paciente y que en ellas influyen distintos factores tanto genéticos como ambientales. La respuesta celular a estos estímulos estresantes no es exclusiva de las neuronas, sino que, como indican numerosos trabajos, el SNC y el sistema inmune se ven afectados de forma similar por procesos dependientes de la EA. Este hecho ha llevado a emplear los linfocitos de pacientes, fácilmente accesibles, como modelo de estudio de las diferentes características fisiopatológicas de la EA. Entre otras, cobran cada vez más importancia las alteraciones en el control del ciclo celular como responsables de la muerte neuronal y del desarrollo de la enfermedad. Por este motivo, la hipótesis de trabajo de nuestro laboratorio se centra en estudiar los mecanismos de regulación del ciclo celular y de la apoptosis en la EA. Para

Objetivos

este trabajo, uno de los modelos de la enfermedad que empleamos son linfocitos inmortalizados de pacientes con EA de aparición tardía y de individuos no afectados de edades similares. Creemos que esta estrategia puede ser de gran interés, además de por la fácil accesibilidad de las células, porque conocer los factores responsables del fallo en los mecanismos de control del ciclo asociados a la enfermedad, puede ser útil para desarrollar posibles estrategias que impidan que las neuronas entren en ciclo, o provocar la detención del mismo en un punto en el que aún sea posible la rediferenciación. Por otro lado, es interesante conocer el proceso de apoptosis en estas células ya que puede ofrecer información que sirva para desarrollar estrategias neuroprotectoras. Por último, sería de gran interés el hallazgo de alteraciones moleculares que fuesen exclusivas de la EA y que pudieran servir de biomarcador para el diagnóstico y seguimiento de la enfermedad.

Además, otro de los modelos de la EA que hemos empleado para la realización de este trabajo son los ratones transgénicos APP/PS1, que expresan mutaciones basadas en la enfermedad familiar y que nos han permitido estudiar simultáneamente las alteraciones del ciclo celular en células periféricas como los linfocitos, y en tejido cerebral, en un intento de demostrar las manifestaciones sistémicas en un modelo murino de la enfermedad y validar el uso de células extraneurales para el estudio de la patología de la EA.

La primera parte de esta tesis se enmarca dentro de trabajos previos del laboratorio, que mostraron alteraciones en la actividad proliferativa y la vulnerabilidad frente a la retirada de suero en linfocitos inmortalizados de pacientes de EA. En concreto, se había descrito una mayor respuesta proliferativa y también una mayor resistencia a la muerte por apoptosis tras la privación de suero en las células de EA, que se relacionaron con alteraciones en los niveles de dos proteínas reguladoras del ciclo celular: p27 en el primer caso y p21 en el segundo (Muñoz y cols., 2008; Bartolomé y cols., 2010). La vía Ca^{2+}/calmodulina (CaM) aparecía como la responsable de la regulación de ambas proteínas, a través de la interacción con otras vías de señalización celular, como la de fosfatidilinositol 3-quinasa/Akt (PI3K/Akt) o la de MAPK. En esta tesis se ha profundizado en el estudio de los mecanismos moleculares que regulan estos procesos.

Los objetivos concretos que se han perseguido son los siguientes:

1.- Estudio de la actividad proliferativa y de la vulnerabilidad frente a la retirada de suero en células extraneurales de pacientes de EA, profundizando en los siguientes aspectos:

1.1.- Regulación del contenido celular de calmodulina.

1.2.- Interacción de la vía Ca^{2+}/CaM con la ruta PI3K/Akt en el control de la actividad

proliferativa.

1.3.- Interacción de la vía Ca^{2+}/CaM con la ruta MAPK para la regulación del contenido celular de p21 en el control de la vulnerabilidad frente a la retirada de suero.

2.- Estudio simultáneo de las alteraciones del ciclo celular en cerebro y células periféricas en el modelo transgénico de EA APP/PS1.

3.- Caracterización por técnicas de Resonancia Magnética de Imagen y de Espectroscopía del ratón APP/PS1.

Materiales y Métodos

3

1 Modelos celulares y animales

El grueso de los materiales y métodos empleados en la realización de este trabajo se exponen en los artículos correspondientes (1-5). En la presente sección describimos con más detalle dos apartados. El primero de ellos se centra en los modelos celulares y animales que hemos empleado en los distintos artículos: por un lado, células periféricas de pacientes (linfocitos inmortalizados con el virus de Epstein-Barr) y por otro lado, un modelo transgénico murino, el APP/PS1. En el segundo apartado se detallan las técnicas empleadas en los estudios de resonancia magnética.

1.1 Células periféricas de pacientes

1.1.1 Obtención de las líneas linfoblásticas

La obtención de células mononucleares de sangre periférica (CMSP) de pacientes e individuos control se realizó separando los componentes de la sangre total por medio de un gradiente de densidad en Ficoll (Lymphoprep™, Axis-Shield PoC AS, Oslo, Noruega). Este método permite separar las células mononucleares por la diferencia de densidad que existe entre éstas y los demás componentes sanguíneos. Las células obtenidas a partir de 10 mL de sangre de cada individuo se cultivaron en suspensión, a una concentración inicial de 1×10^6 células x mL^{-1} en frascos de cultivo de 25 cm^2 en posición vertical. El medio de cultivo utilizado, al que nos referiremos de ahora en adelante como medio de cultivo, fue RPMI-1640 (Invitro-

1 Modelos celulares y animales

gen), suplementado con L-glutamina 2mM, 100 mg/mL de penicilina/estreptomicina (Invitrogen) y, a menos que se indique lo contrario, 10% (v/v) de suero fetal bovino (FBS) (Invitrogen). Las líneas celulares linfoblásticas se establecieron infectando los linfocitos con el virus de Epstein-Barr, obtenido a partir de una línea celular de linfoma de Burkitt (B95-6) cedida por Longina Akhtar (NIH, Bethesda, EEUU). La población de CMSP obtenida anteriormente se puso en cultivo con una alícuota del virus de Epstein-Barr, 5 mg/mL del mitógeno Pokeweed (Sigma), específico de células B, y 1 mg/mL de ciclosporina, para evitar la activación de los linfocitos T presentes en la población que pudieran impedir la proliferación inicial de los linfocitos B infectados. En las siguientes 4 semanas se cambió el medio de cultivo de las células una o dos veces por semana hasta la aparición de los primeros clones linfoblásticos, y, una vez transformadas, las células se dividieron creciendo en suspensión, y cuando se obtuvo la cantidad suficiente se congelaron en nitrógeno líquido, en 1 mL del medio de cultivo antes descrito con 7,5% de dimetilsulfóxido (DMSO). Para trabajar con las líneas linfoblásticas, las células se descongelaron, se retiró el medio con DMSO, se resuspendieron en aproximadamente 8 mL de medio de cultivo, en frascos de cultivo de 25 cm^2 dispuestos en posición vertical y se mantuvieron en un incubador húmedo, con 5% de CO_2 y a 37º C, cambiando el medio de manera rutinaria dos veces por semana.

1.1.2 Características, diagnóstico y selección de los pacientes

Las líneas linfoblásticas empleadas en este trabajo se han obtenido de personas sanas y de personas diagnosticadas de probable enfermedad de Alzheimer de aparición tardía siguiendo los criterios NINCDS-ADRDA. El diagnóstico de los pacientes de EA y la selección de los individuos sanos los llevó a cabo el Dr. Félix Bermejo, del Servicio de Neurología del Hospital Doce de Octubre de Madrid. La extracción de sangre de todos ellos se llevó a cabo en este Servicio, previa firma del consentimiento informado por parte de los donantes o de sus familiares cuando las circunstancias lo requirieron.

Se establecieron aproximadamente 50 líneas linfoblásticas de pacientes de EA esporádica, o de aparición tardía y 50 líneas de individuos no dementes de edad aproximada. Un primer grupo de ellas, englobando 20 pacientes sanos y 20 pacientes de EA, de grado moderado-grave, se empleó para la realización de los experimentos de los artículos 1 y 2 y de los anexos 1 y 3. Posteriormente, se obtuvo un segundo grupo de líneas celulares, que han sido empleadas en los experimentos del artículo 3 y el anexo 2, englobando 23 sujetos sanos y 34 pacientes de EA. Las tablas 2 y 3 recogen las principales características de ambos grupos. La respuesta celular a la estimulación por suero o a la retirada del mismo no se vio afectada por la transformación celular reali-

zada con preparaciones distintas del virus de Espein-Barr.

	Control n=20	EA moderada-grave n=20
Edad (años)	73±3	74±2
Intervalo de edad	(42-89)	(69-91)
Género (V/M)	(11/9)	(5/15)
Duración de la demencia		3,6±3
ApoE 4/3 (nº de casos)	1	6

Tabla 2. Características demográficas de los sujetos implicados en la generación del primer grupo de líneas celulares. La edad se expresa como media±error estándar. V:varón; M:mujer; n: número de sujetos.

	Control n=23	EA leve n=20	EA moderada n=7	EA grave n=7
Edad (años)	75±1	75±2	79±2	76±3
Intervalo de edad	(60-83)	(59-83)	(73-84)	(68-89)
Género (V/M)	(8/15)	(9/11)	(3/4)	(3/4)

Tabla 3. Características demográficas de todos los sujetos implicados en la generación del segundo grupo de líneas celulares. La edad se expresa como media±error estándar. V:varón; M:mujer; n: número de sujetos. Se consideró EA leve cuando la puntuación alcanzada en el test MMSE fue entre 18-24), EA moderada (MMSE entre 10-18) y EA grave (MMSE <10).

1.2 Modelo animal de la enfermedad: ratón APP/PS1

Los ratones empleados en la realización de esta tesis han sido proporcionados por la Dra. Eva Carro, del Laboratorio de Neurociencias del Instituto de Investigación del Hospital Doce de Octubre de Madrid, grupo con el que hemos colaborado en la realización de los artículos 4 y 5.

El modelo empleado, APP/PS1, es un cruce entre el ratón Tg2576, que sobreexpresa la APP humana 695 con la doble mutación sueca

1 Modelos celulares y animales

(K670N, M671L) y el ratón PS1, que porta la mutación (M146L) en *PS1*, ambos basados en el fenotipo C57BJ6. El ratón APP/PS1 resultante se emplea muy habitualmente como modelo transgénico de la EA, ya que expresa suficientes niveles de APP humana y Aβ para garantizar el depósito de amiloide (Antequera y cols., 2009), que se produce de forma progresiva en la corteza y el hipocampo de estos ratones, comenzando a las 8 semanas de edad (Holcomb y cols., 1998; Kurt y cols., 2001). Además, a partir de los 6-9 meses, estos ratones muestran signos de muerte celular por apoptosis en el hipocampo y la corteza (Yang y cols., 2008; Spuch y cols., 2010).

Los animales que hemos empleado tenían una edad de 12 meses, y, como control, hemos utilizado ratones C57BJ6 de la misma edad.

Los estudios de resonancia magnética en los ratones APP/PS1 se llevaron a cabo en el Servicio de Imagen y Espectroscopía por Resonancia Magnética de Alto Campo (SIERMAC), dirigido por el Profesor Sebastián Cerdán, en el Instituto de Investigaciones Biomédicas "Alberto Sols" (CSIC/UAM) de Madrid.

Los experimentos se realizaron en un escáner de resonancia magnética de Alto Campo Bruker Pharmascan® (Burker Medical Gmbh, Ettlingen, Alemania) con un imán horizontal de 7.0 Tesla y 16 cm de diámetro, equipado con un resonador de volumen selectivo para ^{1}H de 23 mm de diámetro y un inserto de gradiente de 90 mm de diámetro (con una intensidad máxima de 30 G/cm). Todos los datos se adquirieron con una consola Hewlett-Packard mediante el programa Paravision v 5.1 operando sobre una plataforma Linux.

Los ratones fueron pre-anestesiados por inhalación empleando un porcentaje de 97%/3% de oxígeno/isofluorano con el animal situado en el interior de una cámara de plexiglass. Posteriormente se introdujo el ratón en el resonador y se mantuvo anestesiado durante todo el ensayo administrando una mezcla de 98%/2% de oxígeno/isofluorano (1 mL/min) a través de una mascarilla nasal. La temperatura del animal se mantuvo constante a 37 ºC con una manta térmica de agua. La tasa respiratoria y la temperatura corporal se monitorizaron durante todo el proceso

2 Técnicas de resonancia magnética

usando un monitor fisiológico Biotrig (Bruker, Medical Gmbh, Ettlingen, Alemania). La figura 21 muestra una foto del proceso. Para cada animal se tomaron diversos tipos de imágenes, mapas y datos que se enumeran a continuación.

Figura 21. Escáner de Resonancia Magnética. A la izquierda y arriba a la derecha se observa el escáner de Resonancia Magnética para pequeños animales que se ha empleado en nuestros estudios. En la imagen ampliada se muestra la disposición del ratón antes de introducirse en el aparato, y se aprecia la mascarilla nasal que le suministra la anestesia durante todo el proceso.

2.1 RESONANCIA MAGNÉTICA DE IMAGEN (MRI)

2.1.1 Obtención de las imágenes

❍ Imágenes pesadas en T2

Adquirimos para cada ratón un total de 16 imágenes pesadas en T2 que empleamos en los estudios estructurales y volumétricos. Las imágenes fueron adquiridas por secuencias de eco de espín rápido con los siguientes parámetros de adquisición: TR = 2500 ms, TE=60 ms, factor RARE =8, FOV = 23mm, matriz de adquisición = 256 x 256, grosor de cada corte = 1 mm, número de cortes 16. Brevemente, cada secuencia se compone de distintos estímulos o pulsos de radiofrecuencia que el tejido absorbe y emite cuando el impulso cesa. Es necesario realizar varios pulsos para poder caracterizar los tejidos, de modo que el tejido emite tras cada pulso una radiofrecuencia que se denomina eco. El parámetro TR se corresponde con el tiempo de repetición, es decir el intervalo transcurrido entre cada pulso de radiofrecuencia mientras que TE es el tiempo de eco, es decir, el intervalo entre la aplicación de cada pulso de radiofrecuencia y la emisión del eco. Estos parámetros se modifican para conseguir contraste entre los tejidos. Valores altos de TR y TE permiten obtener información predominantemente sobre el T2. La matriz de adquisición representa la imagen completa de cada corte y está formada por un determinado número de filas de píxeles. El parámetro campo de visión (FOV, *Field of View*) se relaciona con el tamaño de la imagen, de manera que para obtener una resolución alta interesa que la relación FOV/matriz sea lo menor posible. En general, es necesario realizar una repetición para llenar una fila de píxeles de cada imagen, pero la Adquisición Rápida con Realce de la Relajación (RARE, *Rapid Adquisition with Relaxation Enhancement*) es un tipo de secuencia de eco de espín rápido que permite realizar menos repeticiones para conseguirlo. Un factor RARE de 8 significa que se llenan 8 filas por cada tiempo de repetición.

❍ Mapas de ADC

Los mapas de ADC se obtuvieron a partir de imágenes potenciadas en difusión de agua. Las secuencias de difusión se basan en la secuencia convencional de eco de espín pesada en T2 de Stejeskal-Tanner en la que se aplican gradientes de difusión. En los estudios de difusión se aplican varios valores de b (factor gradiente de secuencia de pulso) con los que se quiere ponderar la imagen, incluyendo un valor b=0, es decir, sin ponderación y se obtiene un grupo de imágenes para cada uno. Los parámetros empleados fueron TR = 3000 ms, TE=51 ms, duración del gradiente de difusión = 16 ms, dirección del gradiente de difusión : izquierda-derecha, FOV = 38 mm, matriz de adquisición = 128 x 128, valores de b = 100,200,300,500,800 y 1200 s/mm^2. Después se obtienen los mapas de ADC, calculando el valor de ADC (expresado en mm^2/s) para cada vóxel, por

2 Técnicas de resonancia magnética

medio de un ajuste lineal de la función $I_b=I_0e^{-ADC\cdot b}$, donde I_b es la intensidad de la señal para un valor b dado e I_0 la intensidad de la señal cuando b=0. El mapa de ADC se representa como una imagen en una escala de colores que refleja la difusión de las moléculas de agua. Obtuvimos un total de 5 cortes para cada ratón.

❍ Mapas de MT

Los mapas de MT se obtuvieron a partir de dos secuencias idénticas de eco de espín, en las que se aplicaba, M(t) , o no ,M(o), un pulso de presaturación MT de 8.0 ppm. Los parámetros empleados fueron los siguientes: TR = 2500 ms, TE=9,8 ms, FOV = 20mm, matriz de adquisición 128x128. Los mapas M(t)/M(o) se prepararon dividiendo pixel a pixel las intensidades de las imágenes saturadas y no saturadas. Obtuvimos un total de 5 cortes para cada ratón.

2.1.2 Procesamiento de las imágenes

Las imágenes pesadas en T2 se emplearon para examinar las diferentes estructuras cerebrales y realizar estudios volumétricos. Las regiones de interés se delinearon superponiendo sobre la imagen pesada en T2 la imagen anatómica correspondiente obtenida del atlas del cerebro del ratón "The Mouse Brain in Stereotaxic Coordinates" (Franklin y Paxinos, 1997). Como referencia común entre el atlas y la imagen en T2, se seleccionó la marca anatómica del tercer ventrículo dorsal (Bregma -1.46 mm, Interaural 2.34 mm). En la figura 22 se puede ver un ejemplo del proceso. Una vez delimitadas las áreas de interés, se calculó para cada una de ellas su área y la intensidad de la señal de T2 correspondiente empleando el programa Image J (NIH, Bethesda, Maryland, EEUU).

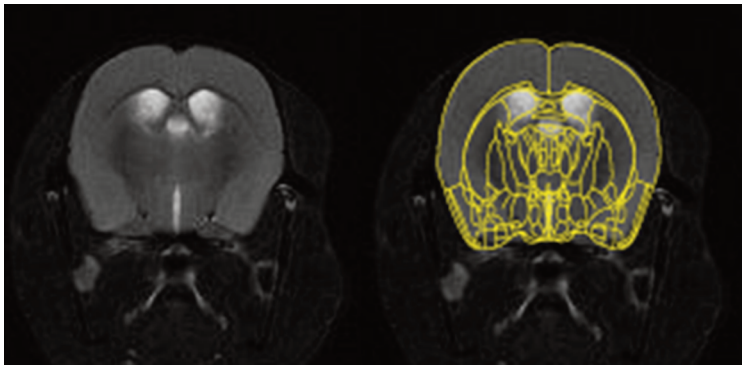

Figura 22. Superposición de la imagen del anatómica del atlas sobre la imagen pesada en T2 para la determinación de las áreas cerebrales de interés.

El mismo proceso se siguió con los mapas paramétricos ADC y MT. En este caso, se emplearon las imágenes pesadas en T2 con la misma geometría que los mapas ADC y MT para determinar la imagen correspondiente del atlas Paxinos, y posteriormente ésta se superpuso en los mapas ADC y MT para calcular los valores de ADC y %MT en cada región de interés. Se puede observar una imagen de ambos mapas en la figura 23. Como se explicó en la introducción, los mapas de ADC representan la difusión del agua en el tejido cerebral. Una mayor difusión de agua aparece en tonos más rojos. Por su parte, en los mapas de MT se estudia la relación entre los protones libres y los unidos a macromoléculas. El %MT se usa como parámetro de medida del fenómeno y depende del entorno químico y biofísico que rodea las macromoléculas y sirve por lo tanto para detectar y cuantificar cambios histológicos que acompañan la enfermedad (Ridha y cols., 2007). En este caso, el agua aparece en tono azul.

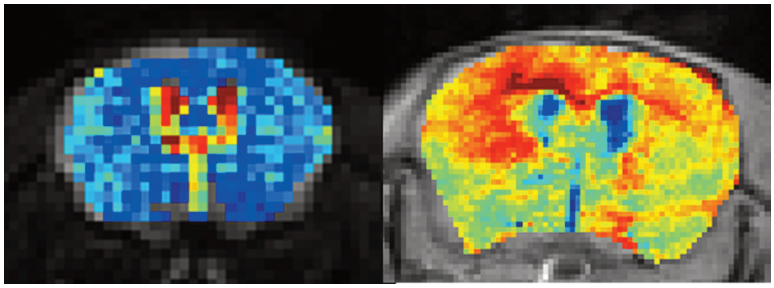

Figura 23. Ejemplos de mapa de ADC (izquierda) y MT (derecha) en un ratón control. En ambos casos se representa el mismo corte anatómico. En el caso de los mapas de ADC, las zonas con más difusión de agua se muestran rojas mientras que en los mapas de MT , las zonas de mayor contenido acuoso aparecen azules.

2.2 Resonancia magnética de espectroscopia

Los ensayos de espectroscopía de hidrógeno en vivo se realizaron en el mismo aparato que la MRI y bajo las mismas condiciones. Se adquirieron en todos los casos espectros empleando una secuencia PRESS *(Point Resolved SpectroScopy)* con una secuencia VAPOR para suprimir la señal de agua y se emplearon los siguientes parámetros de adquisición: TR= 3000 ms, TE=35 ms, 128 adquisiciones, tamaño del vóxel = 3 mm^3. Las regiones estudiadas fueron el área cortical y el área subcortical, y en cada una de ellas se analizaron los metabolitos NAA, Cr y Cho. Sus concentraciones se cuantificaron usando el programa LCModel (Provencher, 1993). En todos los casos, los valores de dichos metabolitos se encontraban por debajo del 20% de la cota inferior de Cramér-Rao (CRLB, *Cramér-Rao Low Bound*) un estimador de la desviación estándar del ajuste de cada metabolito. Para las comparaciones entre ratones control y APP/PS1, las concentraciones de NAA y Cho se normalizaron respecto a Cr. Aunque se in-

vestigaron más parámetros como lactato, glutamato, glutamina, mioinositol o taurina con el programa LCModel, no se tuvieron en cuenta por estar por encima del 20% de CRLB.

Resultados

4

1 Presentación de los resultados

Los resultados de esta tesis se presentan en Formato Publicaciones, y se encuentran descritos en los siguientes artículos:

Artículo #1: Natividad de las Cuevas, Úrsula Muñoz, Fernando Bartolomé, **Noemí Esteras**, Carolina Alquézar, Ángeles Martín-Requero (2010). "Cell cycle and Alzheimer's Disease. Studies in non-neuronal cells". **J Applied Biomed** 8:121-130. Review.

Artículo #2: **Noemí Esteras**, Úrsula Muñoz, Carolina Alquézar, Fernando Bartolomé, Félix Bermejo-Pareja , Ángeles Martín-Requero (2012). "Altered calmodulin degradation and signaling in non-neuronal cells from Alzheimer's disease patients." **Curr Alzheimer Res** 9(3): 267-277.

Artículo #3: **Noemí Esteras**, Carolina Alquézar, Félix Bermejo-Pareja, Emilia Bialopiotrowicz, Urszula Wojda, Ángeles Martín-Requero (2012). "Downregulation of ERK1/2 activity by CaMKII modulates p21 and survival of immortalized lymphocytes from Alzheimer's disease patients". Submitted.

Artículo #4: **Noemí Esteras**, Fernando Bartolomé, Carolina Alquézar, Desiré Antequera, Úrsula Muñoz, Eva Carro, Ángeles Martín-Requero

1 Presentación de los resultados

(2012). "Altered cell cycle-related gene expression in brain and lymphocytes from a transgenic mouse model of Alzheimer's disease (APP/PS1)" **Eur J Neurosci**. doi:10.1111/j.1460.9568.2012.08178.x

Artículo #5: **Noemí Esteras**, Carolina Alquézar, Fernando Bartolomé, Desiré Antequera, Laura Barrios, Eva Carro, Sebastián Cerdán, Ángeles Martín-Requero (2012). "Systematic Evaluation of Magnetic Resonance Imaging and Spectroscopy Techniques for Imaging a Transgenic Model of Alzheimer's Disease (AbetaPP/PS1)." **J Alzheimers Dis** 30(2): 337-353.

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Resumen

Esta revisión recapitula el trabajo realizado en nuestro laboratorio centrado en el papel de las alteraciones del ciclo celular en la patogénesis de la EA en células periféricas de pacientes. La reactivación de la maquinaria del ciclo en neuronas postmitóticas es un evento temprano que se relaciona con la muerte neuronal y con el desarrollo de la enfermedad. Estas características también están presentes en células periféricas de pacientes, como linfocitos o fibroblastos. Por estos motivos, la hipótesis de trabajo de nuestro laboratorio se centra en estudiar los mecanismos moleculares que regulan los procesos de proliferación y supervivencia/muerte celular en linfocitos inmortalizados de pacientes de alzhéimer de aparición tardía.

Los resultados que aquí se exponen muestran que los linfocitos de pacientes de EA presentan, en presencia de suero, una mayor actividad proliferativa que las células control y, en ausencia de suero, una mayor resistencia a la apoptosis inducida por la ausencia de factores tróficos. Ambas características dependen en último lugar de dos proteínas reguladoras del ciclo celular, p27 en el primer caso y p21 en el segundo. La vía Ca^{2+}/CaM interacciona con otras rutas para regular los niveles de ambas proteínas en estas células. En el primer caso, la ruta Ca^{2+}/CaM sobreactiva la vía PI3K/Akt, lo que favorece la fosforilación de p27 y su degradación en el proteasoma. La degradación de p27, que actúa como inhibidor del ciclo, induce un aumento de la actividad del complejo Ciclina E/Cdk2, que aumenta la fosforilación del proteína del retinoblastoma y libera el factor E2F favoreciendo la progresión del ciclo celular.

En el segundo caso, la retirada de suero induce la activación dependiente de Ca^{2+}/CaM de CaMKII. Este hecho se acompaña de una disminución de la actividad de la ruta de las MAPK (ERK1/2), de un aumento en los niveles de p21 y de una mayor resistencia a la apoptosis.

Por estos motivos, las características neoplásicas que muestran las células de los pacientes de EA pueden ser consideradas manifestaciones sistémicas de la enfermedad.

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REVIEW

Cell cycle and Alzheimer's disease: studies in non-neuronal cells

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Summary
The most common cause of dementia in the elderly is Alzheimer disease (AD). In Europe, AD is a leading cause of death. The prevalence of this disease in developed countries is increasing because of very significant shifts in life expectancy and demographic parameters. AD is characterized by progressive cognitive impairment, resulting from dysfunction and degeneration of neurons in the limbic and cortical regions of the brain. Two prominent abnormalities in the affected brain regions are extracellular deposits of β-amyloid, and intracellular aggregates of tau protein in neurofibrillary tangles. The role of these features in AD pathogenesis and progression is not yet completely elucidated. Research over the last decade has revealed that the activation of cell cycle machinery in postmitotic neurons is one of the earliest events in neuronal degeneration in AD. Here we summarize evidence to support the hypothesis that cell cycle alterations occur in cells other than neurons in AD sufferers. Immortalized lymphocytes from AD patients have show an enhanced rate of proliferation associated with G1/S regulatory failure induced by alterations in the cyclin/CDK/pRb/E2F pathway. In addition, these cells have a higher resistance to serum deprivation-induced apoptosis. These neoplastic-like features, cell cycle dysfunction and impaired apoptosis can be considered systemic manifestations of AD disease.

Key words: Alzheimer's disease; lymphocytes; cell cycle; cell survival; p27; p21; calmodulin; PI3K/Akt; ERK1/2

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting aged people; AD prevalence is approximately 1% between 65 and 69 years and is higher than 50% in individuals above 95 years. The predominant clinical manifestation is memory loss, but a number of other changes in brain functioning, including impairments in language and visual-spatial skills, and disorientation also characterize this disease. With increasing life expectancy across the world, dementia is a rapidly growing socioeconomic and medical problem. The hallmarks for AD are β-amyloid plaques, neurofibrillary tangles, and regionalized neuronal loss. However our understanding of the role that these features of AD play in the etiology of the disease remains incomplete.

AD is extremely complex and genetically heterogeneous. The majority of patients with the so-called "sporadic" disease exhibit clinical signs during the seventh decade, whereas individuals with inherited AD (FAD) often become demented in mid

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life. To date autosomal dominant FAD has been linked to the presence of mutations in the genes *APP*, *PS1*, and *PS2*, which are genes encoding the amyloid precursor protein (APP), located in chromosome 21 or the presenilins PS1 or PS2 in chromosomes 14 and 1 respectively. The late-onset or sporadic AD has been associated with genetic factors modifying the risk of suffering AD. Notably, the risk of *apoE* allele type is a susceptibility locus with *apoE4* showing a dose-dependent contribution to AD (Strittmatter et al. 1993). Various methods of genetic analysis indicate that additional genes predisposing to AD exist. A complete list of susceptibility genes associated with increased risk for AD can be found in http://alzgene.org (Bertram et al. 2007).

CELL CYCLE AND AD

Despite progress in uncovering many of the factors that contribute to the etiology of this disease, the cause of neuronal death is largely unknown. One promising theory to explain neurodegeneration in AD is that neuronal loss could be associated with cell cycle disturbances. This hypothesis receives support from evidence that a variety of cyclins and cyclin-dependent kinases (CDKs) are up-regulated in the brain of AD patients (Nagy et al. 1998), suggesting that elements that normally control cell cycle progression in proliferating cells may modulate neuronal death as well. Hippocampal and selected cortical neuronal populations in AD exhibit phenotypic changes characteristic of cells re-entering the cell division cycle (Arendt 2003), and it has been further demonstrated that a significant fraction of the hippocampal pyramidal and basal forebrain neurons have fully or partially replicated four independent loci of three different chromosomes (Yang et al. 2001). The successful duplication of DNA indicates that some neurons had completed the S phase of cell cycle (Mosch et al. 2007). These anomalies were not found in unaffected regions of AD brains or in the hippocampus of non-demented age-matched controls. Recent work has shown that cell cycle-related events are present many months before the appearance of plaques or signs of inflammation in brain of murine models of AD (Yang et al. 2006), as well as in brain of patients with Mild Cognitive Impairment (MCI) (Ueberham and Arendt 2005). MCI is a risk stage for development of AD within the next 3–5 years. Therefore, it appears that cell cycle dysfunction is implicated in disease onset and early development and it seems to confer selective vulnerability to neurons (Webber et al. 2005). Importantly, most of the AD pathological features, including β-amyloid, tau, presenilin mutations, oxidative stress, dystrophic neuritis, DNA damage and aneuploid are somehow related to cell cycle control, providing a link between cell cycle disturbances and neurodegeneration (Zhu et al. 2007, Żekanowski and Wojda 2009). A causal relationship between cell cycle re-entry and neurodegeneration has been recently reported in a transgenic mouse model in which conditional and neuron-specific expression of the proto-oncogene *c-Myc* leads to cognitive deficit and neurodegeneration (Lee et al. 2009a). It is now assumed that adult neurons, rather than staying permanently postmitotic, must constantly keep their cell cycle in check (Herrup and Yang 2007). If control of neuronal cell cycle fails, the consequence is the entrance of neurons into an altered and vulnerable state, often leading to death (Zhu et al. 2007). Based on these findings, some authors have come to consider AD disease as an abortive neoplastic disorder, that is, a disease of the cell cycle, and thus, the knowledge of the intimate involvement of cell-cycle checkpoints in molecular pathogenesis of AD might be important for diagnostic purposes and particularly in the search for treatment strategies (see Lee et al. 2009b for a review).

MITOTIC SIGNALS

The search for factors responsible for the formation of neurofibrillary tangles and amyloid deposits has yielded several clues to the hypothesis that cell cycle-related phenomena is implicated in the accumulation of AD pathology. CDKs are involved in the phosphorylation of tau (Weishaupt et al. 2003), the main component of tangles as well as a key protein for cytoskeleton organization that occurs during neurite outgrowth and perhaps in aberrant neuronal sprouting (Webber et al. 2005). β-amyloid has been identified as mitogenic *in vitro* (Milward et al. 1992). APP may induce the activation of cell cycle proteins in neurons (Neve and McPie 2006). Conversely, cell cycle proteins that normally control the progression of cell cycle at the G1/S checkpoint are present in tangle bearing neurons (McShea et al. 2007). The accumulation of potentially mitogenic growth factors (EGF, bFGF) in diffuse amyloid deposits could represent the trigger that initiates the re-entry of neurons into the cell cycle (McShea et al. 1999). DNA damage induced by oxidative stress has been associated with overexpression of the tumor suppressor protein p53 and cell cycle reentry-induced apoptosis in cultured neurons (Kruman et al. 2004).

p53 could activate cell cycle or apoptosis depending on the success of the DNA repairing process (Żekanowski and Wojda 2009). Other factors, including many of the identified risk factors for Alzheimer's disease, such as elevated plasma homocysteine levels, ageing, menopause, low thyroid levels, low level prolonged oxidative stress or head injury, can either represent mitogenic signalling for neurons or facilitate cell cycle re-entry in vulnerable neuronal populations (reviewed by Arendt 2003).

CELLULAR AND ANIMAL MODELS

Limitations in the use of the postmortem brain for examining molecular mechanisms underscore the need to develop cell or animal models representative of the pathogenesis that characterize AD. Thus, there has been a strong impetus in the last decade to develop a number of different transgenic mouse models of AD that overexpress the human FAD genes in the context of the mouse. This has proven to be a valuable resource in the study of APP processing and in the exploration and design of disease therapies (Morrissette et al. 2009). The AD mice show microglial activation, astrocytosis, and changes in neuronal cytoskeleton proteins including tau. Many of these model organisms have also been shown to have significant memory deficits. Although most of these mice don't show significant loss of neuronal bodies, cell cycle-related events appear to occur also in the mouse brain. Recently, it has been demonstrated in four different plaque bearing mice, that neurons in the most vulnerable areas have begun a true cell cycle (Yang et al. 2006).

An alternative strategy to study the pathogenesis of AD is the use of non-neuronal cells from patients. Numerous observations indicate that, while the predominant clinical expression arises from brain, AD has systemic expression at the cellular and molecular levels. Considerable precedent exists for studying AD with peripheral tissues, including lymphocytes, fibroblasts, and platelets (Etcheberrigaray and Ibarreta 2001, Casoli et al. 2008). The use of peripheral tissues complement studies of autopsy samples and provide a useful tool to investigate dynamic processes such as signal transduction mechanisms, oxidative metabolism, etc.

CELL CYCLE DISTURBANCES IN NON-NEURONAL CELLS

Work from our laboratory and others have shown cell cycle disturbances in peripheral cells, such as lymphocytes from AD patients, suggesting that dysfunction of the cell cycle is a more general phenomenon affecting cells other than neurons. Immortalized lymphocytes from AD patients showed altered response to mitogenic stimulation relative to control subjects (Urcelay et al. 2001, Nagy et al. 2002). Fibroblasts collected from AD patients also show an aberrant cell cycle-dependent Ca^{2+} response (Tatebayashi et al. 1995). We, and others have also found failure of the G1/S transition checkpoint, similar to that reported in AD brain, in lymphocytes from AD subjects (Nagy et al. 2002, de las Cuevas et al. 2003), and interestingly in MCI patients as well (Nagy et al. 2002, Zhou and Jia 2010).

We also found that lymphocytes from AD patients were more resistant to serum withdrawal-induced cell death (de las Cuevas et al. 2005), suggesting that control of cell fate depending on the presence or absence of growth stimulatory signals is impaired in peripheral cells from AD sufferers. These features might represent an adaptative response for AD cells that are exposed to chronic stress. It has been considered that susceptible neurons in AD survive for long time in a compromised way by delaying the apoptotic process, a mechanism termed abortosis or abortive apoptosis (Jellinger 2006).

PROLIFERATIVE ACTIVITY OF IMMORTALIZED LYMPHOCYTES FROM CONTROL AND AD PATIENTS

To investigate the distinct cell cycle regulation in AD at the systemic level, we performed a comparative study on cell proliferation, cell cycle profiles, and expression levels of key cell cycle regulatory proteins in lymphoblasts derived from control and late-onset AD subjects. These lymphoblastoid cell lines, obtained by infecting peripheral blood mononuclear cells with the Epstein Barr virus, retained the cellular response of freshly obtained lymphocytes, to serum addition or withdrawal (Bartolomé et al. 2007, Muñoz et al. 2008a).

Lymphoblasts from AD patients exhibited a serum dose-dependent enhanced rate of proliferation compared with cells from normal age-matched controls (Urcelay et al. 2001, de las Cuevas et al. 2003). AD lymphoblasts show cell cycle progress modifications such as a decrease of cells in G1, and an increased number of cells in S phase, together with altered expression and phosphorylation of several proteins involved in regulation of the G1/S transition check point (de las Cuevas et al. 2003, 2005, Muñoz et al. 2005). AD lymphoblasts showed increased

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phosphorylation of the retinoblastoma protein (pRb) and other members of the family of pocket proteins compared with cell lines derived from normal age-matched controls (de las Cuevas et al. 2003, Muñoz et al. 2005). pRb is sequentially phosphorylated by two sets of protein kinases, the cyclinD/CDK4 and cyclin E/CDK2 complexes (Mittnacht 1998). The activity of the latter, was found to be enhanced in AD lymphoblasts (Muñoz et al. 2008a). Furthermore, we demonstrated that the increase in CyclinE/CDK2 activity was not due to changes in the expression levels of either cyclin E or CDK2, but rather, to the decreased levels of the CDK inhibitor p27 found in AD lymphoblasts (de las Cuevas et al. 2003, Muñoz et al. 2005).

Once pRb-related proteins are phosphorylated, the transcription factor E2F is released and activated (Weinberg 1995). Accordingly, nuclear extracts from AD lymphoblasts showed reduced E2F-DNA binding activity as determined by EMSA analysis (de las Cuevas et al. 2005). In contrast the activity of NF-κB was found to be decreased in lymphocytes from AD patients, and was not related to the serum-induced enhanced proliferation, but associated instead with decreased vulnerability of AD cells to serum deprivation (de las Cuevas et al. 2005).

Two different reports have shown that freshly obtained lymphocytes from AD patients are less sensitive to G1/S transition blockers, thus suggesting a failure of the G1/S checkpoint function (Nagy et al. 2002, Zhou and Jia 2010). These authors also found these cell cycle alterations in MCI or mild-AD patients.

Further work focused in delineating the molecular mechanisms underlying the p27 down-regulation in AD cells. It was found that this effect was due to increased p27 degradation. A shorter half-life of the p27 protein was detected in AD lymphoblasts as compared with control cells (Muñoz et al. 2008a). p27 proteolysis is a three-step process that requires phosphorylation at Thr187, recognition by the F-box protein SKP2, ubiquitination, and degradation by the 26S proteasome. Increased phosphorylation of p27 protein at Thr187 in AD cells, rather than changes in the 26S proteasome machinery, seems to account for decreased p27 levels. An inverse relationship between phospho-p27 and p27 content was found, while total proteasome activity and accumulation of ubiquitin-tagged proteins did not change significantly (Muñoz et al. 2008a, b).

Interestingly, the enhanced proliferative activity and changes in cell cycle regulatory proteins, can be modulated pharmacologically by treating AD cells with the anti-inflammatory cyclopentenone 15-deoxy-prostaglanding J_2 or simvastatin (Muñoz et al. 2005, 2008b, Sala et al. 2008). Therefore these observations provide a plausible explanation for the reported apparent benefits of these drugs preventing or delaying the clinical features of AD (Stewart et al. 1997, Wolozin et al. 2000).

VULNERABILITY OF CONTROL AND AD LYMPHOBLASTS TO SERUM DEPRIVATION

Lymphoblasts from AD subjects were found to be more resistant to serum withdrawal (de las Cuevas et al. 2005, Bartolomé et al. 2007). In control cells, there was a progressive appearance of cell death after 24 h of serum starvation. However, little cell death, as assessed by decreasing levels of MTT reduction was observed in AD cells even after 96 h of serum deprivation (de las Cuevas et al. 2005, Bartolomé et al. 2007). Selective impairment of the mechanisms involved in cell death has been also reported in fibroblasts from AD patients. The protective mechanism of AD fibroblasts against H_2O_2 was related to an impairment of cell cycle arrest and a diminished induction of apoptosis (Uberti et al. 2002).

The lower sensitivity of AD lymphoblasts to serum withdrawal was associated with changes in the balance of pro- and anti-apoptotic proteins. Moreover it was shown that the survival of AD cells was accompanied by enhanced p21 content as compared with that of control cells (Bartolomé et al. 2009b). A number of recent studies pointed out that in addition to being an inhibitor of cell proliferation, p21 may protect cells from apoptosis (Gartel and Radhakrishnan 2005). For example, it has been reported that up-regulation of p21 blocked the oxidative stress-induced death of human myeloma U266 cells (Kim et al. 2001) and that inducible expression of exogenous p21 render glioblastoma cells resistant to chemotherapy drugs (Ruan et al. 1998). Thus the increase in p21 cellular content in AD lymphoblasts may confer these cells a survival advantage.

SIGNALLING PATHWAYS AND MEDIATORS INVOLVED IN INCREASED PROLIFERATION AND SURVIVAL OF AD LYMPHOCYTES

Since dysregulation of calcium homeostasis is among the major cellular alterations in AD (Thibault et al. 2007) we investigated whether alterations in the

major cellular Ca^{2+}-binding protein, calmodulin (CaM) were involved in the altered cellular response of AD lymphoblasts. We found that two structurally unrelated antagonists of CaM, like calmidazolium (CMZ) and W-7, inhibited the proliferation of lymphoblasts exclusively from AD patients (Urcelay et al. 2001, de las Cuevas et al. 2003).

The CaM antagonists were also able to revert the resistance of AD lymphoblasts to cell death induced by serum deprivation (de las Cuevas et al. 2005, Bartolomé et al. 2007). Therefore, CaM seems to play a pivotal role in transmitting proliferative/survival signals from the plasma membrane to the nucleus. Whether CaM contributes to cell proliferation or apoptosis may depend on cellular CaM levels and/or activity, as well as the presence of growth-stimulatory signals.

The combination of CaM antagonist and specific inhibitors of intracellular pathways potentially implicated in the regulation of cell proliferation and apoptosis, revealed the interaction of Ca^{2+}/CaM with PI3K/Akt and ERK1/2 pathways in the presence or in the absence of serum, respectively. Table I shows how, in contrast to the selective effect of CMZ, preventing the enhanced stimulation of AD cells, Ly294002, the inhibitor of PI3K/Akt, decreased proliferation of both control and AD lymphoblasts. SB202190, the inhibitor of p38, and PD98059, inhibitor of ERK1/2 had no effect on cell proliferation. However, treatment of control cells with PD98059 prevented cell death induced by serum starvation (Table I). This inhibitor had no effect in AD cells, but blunted the effects of CMZ inducing apoptosis in these cell lines. PI3K/Akt activity, as assessed by increased Akt phosphorylation, was found to be enhanced in AD cells following serum stimulation, compared with the activity observed in control cells (Muñoz et al. 2008a, Bartolomé et al. 2009a, b). In contrast a reduced sustained phosphorylation of ERK1/2 was observed in AD cells upon serum deprivation (Bartolomé et al. 2007). Both overactivation of PI3K/Akt and downregulation of ERK1/2 pathways in AD cells were prevented by CaM antagonists (Bartolomé et al. 2007, Muñoz et al. 2008a).

The Ca^{2+}/CaM dependent overactivation of PI3K/Akt appears to be the upstream event in the serum-mediated enhanced proliferation of AD lymphoblasts. The CaM antagonists and the inhibitor of PI3K/Akt have similar effects in p27 phosphorylation at Thr187, and protein degradation (Muñoz et al. 2008a). It was reported that overactivation of PI3K/Akt in AD cells may favor the CyclinE/CDK2-mediated phosphorylation of p27 at Thr187. In addition, PI3K/Akt phosphorylate other p27 residues (Thr157, Thr159), which are important in controlling the nucleo-cytoplasmic traffic of p27 (Liang et al. 2002). The exclusion of p27 from the nucleus would then facilitate its degradation by the proteasome (Muñoz et al. 2008a,b) and thus relieve cyclinE/CDK2 kinase activity from p27 inhibition. In fact, it was observed that pRb hyperphosphorylation, E2F, and cyclinE/CDK2 activities, as well as p27 content, were all affected by CaM antagonists (de las Cuevas et al. 2003, 2005, Muñoz et al. 2008a, b). On the other hand, the Ca^{2+}/CaM dependent down regulation of ERK1/2 seems to be the key event in protecting AD cells from serum deprivation-induced cell death (Bartolomé et al. 2007). By decreasing the activity of ERK1/2, it induces the overexpression of p21 protein, which in turn blocks the serum withdrawal-induced apoptosis (Bartolomé et al. 2009b). Interestingly, it was found that the CaM binding proteins CaMKII and calcineurin are not involved in the serum-enhanced proliferation of AD lymphoblasts (de las Cuevas et al. 2003, Muñoz et al. 2008a). However CaMKII seems to play an important role in controlling cell survival under serum deprivation, since either CaM antagonists or the CaMKII inhibitor KN-62 sensitize AD cells to death triggered by the absence of growth stimulatory signals (Bartolomé et al. 2007). CaM antagonists had no effect in control cells, suggesting a threshold for CaM activation as the survival signal (de las Cuevas et al. 2005, Bartolomé et al. 2007, Muñoz et al. 2008a). In fact, higher CaM content was found in lymphoblasts from AD patients (Muñoz et al. 2008b).

As reported for other cell types (Pérez-García et al. 2004), we were able to observe that CaM binds to the p85α subunit of PI3K (Muñoz et al. 2008b). Therefore, CaM could contribute to PI3K overactivation in AD cells through this mechanism, as association of CaM with the SH2 domain in p85 leads to PI3K activation (Pérez-García et al. 2004).

The mechanism(s) by which CaM downregulates the ERK1/2 pathway in AD lymphoblasts is not yet known, thought a mechanism of association of CaMKII with Ras-Raf-1/MEK/ERK1/2 seems likely. This issue is currently under investigation in our laboratory.

In summary, our work revealed a functional relationship between Ca^{2+}/CaM and PI3K/Akt or ERKs in serum-induced signalling in immortalized lymphocytes, controlling cell fate (proliferation/death or survival) depending on growth factor availability. The proposed scenario is represented schematically in Fig. 1.

Finally, it is worth mentioning that a deregulation of both PI3K/Akt and ERK1/2 signalling pathways has been reported in AD brains. Increased

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Table I. **Influence of signalling pretubation on cell proliferation/cell death.**

Treatment	Control	AD
	Inhibition of cell proliferation (%)	
+10% FBS	–	–
20 μM Ly294002	35 ± 3	41 ± 5
20 μM PD98059	2 ± 4	8 ± 5
20 μM SB202190	6 ± 4	5 ± 3
1 μM CMZ	4 ± 2	30 ± 3*
1 μM CMZ + 20 μM Ly294002	33 ± 4	36 ± 5
	Cell survival (%)	
–10% FBS	69 ± 1	92 ± 5‡
20 μM Ly294002	71 ± 4	87 ± 2
20 μM PD98059	96 ± 2‡	92 ± 9
10 μM SB202190	66 ± 6	87 ± 6
1 μM CMZ	60 ± 3	61 ± 6
1 μM CMZ + 20 μM PD98059	93 ± 6‡	87 ± 6

Lymphoblasts from control and AD individuals were seeded at an initial density of 1×10^6/ml on day 0, in the presence or in the absence of FBS, and treated with the indicated concentrations of drugs for 3 days. Cell survival was expressed as % of cells at day 0. Values shown are the mean 6 SEM for 4–6 observations.
* $p<0.05$ significantly different from control cells; ‡ $p<0.05$ significantly from untreated AD cells.

phospho-Akt (Ser473) has been detected in AD temporal cortex, accompanied by increased levels of phosphorylation of Akt substrates such as GSK3, mTOR, tau and lower levels of p27 (Griffin et al. 2005). Increased phosphorylated p27 (Thr 187) has been also found in AD brains. Importantly, Thr187-p27 shows a considerable overlap with tau-positive neurofibrillary pathology, including neurofibrillary tangles and dystrophic neuritis (Ogawa et al. 2003). On the other hand, ERK1/2 activation has been shown in degenerative neurons in close association with neurofibrillary tangles, suggesting the implication of this pathway in AD pathogenesis (Knowles et al. 1999).

Thus, our results obtained in peripheral cells from AD patients, demonstrated that dysfunction of PI3K/Akt, and ERK1/2 signalling also occur outside the CNS, supporting the hypothesis that AD has systemic expression at cellular and molecular levels. Therefore, peripheral cells from patients may be a potential useful surrogate for diagnosis, prognosis and therapeutic monitoring of AD.

While most of the studies on AD lymphocytes had been focused on detecting disease-specific changes that may serve as biomarkers, the clinical consequences, if any, of the enhanced proliferative response of lymphocytes in AD patients, remain to be established, as well as the role that they may play in the chronic inflammation associated with this disease.

CONCLUSIONS

Cell cycle disturbances are evident in non-neuronal cells from AD patients. While the precise origins of cell cycle alterations are not fully understood, a complex interaction of Ca^{2+}/CaM with the PI3K/Akt

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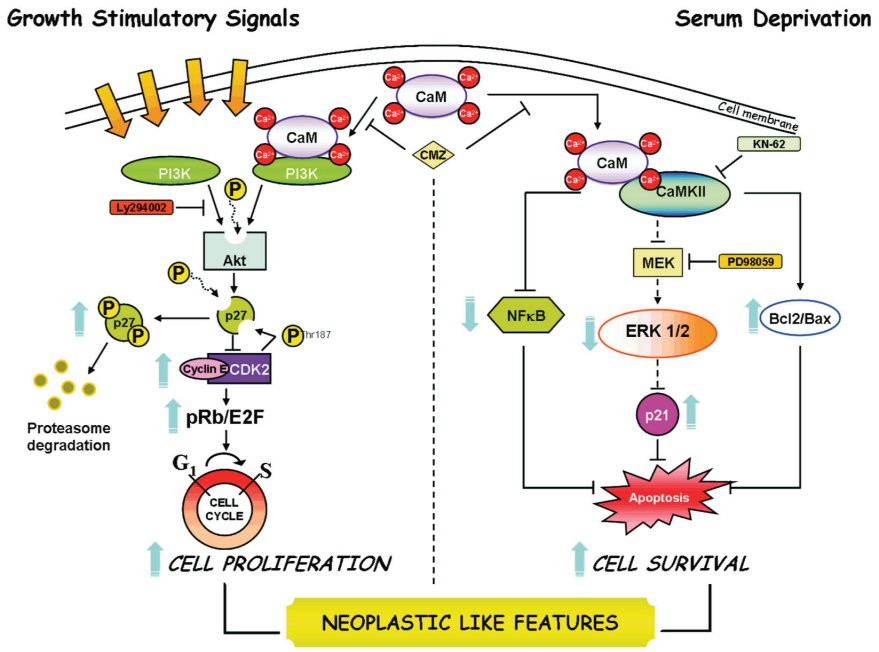

Fig. 1. **Depiction of the proposed signalling pathways altered in AD lymphoblasts.** In AD cells, increased levels of CaM synergise with serum stimulation and promote overactivation of PI3K/Akt leading to enhanced p27 degradation, and activation of cyclin/CDK/pRb/E2F, therefore favouring, the progression of cells through the cell cycle. In the absence of serum, the Ca^{2+}/CaM-binding protein, CaMKII, decreases the serum-deprivation induced NF-κB and ERK1/2 activation in comparison with control cells, and increases the cellular content of p21, which then seems to protect AD lymphoblasts from apoptosis induced by the absence of trophic support.

and ERK1/2 pathways seems to be the master regulator of cell survival, controlling cell proliferation or preventing apoptosis depending on growth conditions. Two cell cycle regulatory proteins, the CDK inhibitors, p27 and p21, are ultimately responsible for the enhanced proliferation and increased resistance to cell death, respectively. Whereas downregulation of p27 in a Ca^{2+}/CaM-dependent manner induces the enhanced proliferative response of immortalized lymphocytes from AD patients, upregulation of p21 seems to help AD cells to escape from serum deprivation-induced apoptosis. The distinct cell cycle and apoptosis control in lymphoblastoid cells from AD patients offer a noninvasive tool for investigating the pathogenesis of AD and suggest a number of molecular targets for potential AD therapies.

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3 Alteración de la degradación y señalización de calmodulina en células periféricas de pacientes de alzhéimer

Artículo #2 : **Noemí Esteras**, Úrsula Muñoz, Carolina Alquézar, Fernando Bartolomé, Félix Bermejo-Pareja , Ángeles Martín-Requero (2012). "Altered calmodulin degradation and signaling in non-neuronal cells from Alzheimer's disease patients." **Curr Alzheimer Res** 9(3): 267-277.

Resumen

Trabajos previos de nuestro laboratorio habían mostrado que el control de la proliferación y la supervivencia de los linfocitos inmortalizados de pacientes de EA dependían en último lugar de alteraciones en la vía de señalización dependiente de Ca^{2+}/CaM. Por este motivo, en este trabajo se ha investigado la regulación de los niveles y la actividad de calmodulina en los linfoblastos de pacientes de EA. En primer lugar, observamos un aumento en el contenido de CaM en las células de pacientes. El aumento en los niveles celulares de la proteína no se debe a una mayor expresión de ninguno de los tres genes que la codifican, como muestran las PCR cuantitativas en tiempo real (qRT -PCR) de los genes *CALM1, 2 y 3*. Sin embargo, la vida media de CaM es mayor en linfoblastos de pacientes de EA que en controles, sugiriendo que la degradación de la proteína está alterada en las células de pacientes. La tasa de degradación de calmodulina depende de los niveles intracelulares de calcio y de ROS, que aparecen elevados en los linfocitos de EA. Además, la degradación de calmodulina se produce a través del sistema ubiquitina-proteasoma. Los niveles elevados de CaM se asocian con una mayor actividad de la misma, como muestran la sobreactivación de la vías CaMKII y PI3K/Akt. En relación con esta última, nuestros resultados indican que el aumento en los niveles de CaM coopera con el suero para sobreestimular la vía PI3K/Akt, a través de la unión directa de CaM a la subunidad reguladora (α-p85) de la PI3K. Estos resultados sugieren que el fallo a nivel sistémico del control de la degradación de CaM, y por lo tanto, de la señalización celular que depende de la vía Ca^{2+}/CaM, puede ser un mecanismo importante en la patogénesis de la EA.

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Altered Calmodulin Degradation and Signaling in Non-Neuronal Cells from Alzheimer's Disease Patients

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Abstract: Previous work indicated that changes in Ca^{2+}/calmodulin (CaM) signaling pathway are involved in the control of proliferation and survival of immortalized lymphocytes from Alzheimer's disease (AD) patients. We examined the regulation of cellular CaM levels in AD lymphoblasts. An elevated CaM content in AD cells was found when compared with control cells from age-matched individuals. We did not find significant differences in the expression of the three genes that encode CaM: CALM1, 2, 3, by real time RT-PCR. However, we observed that the half-life of CaM was higher in lymphoblasts from AD than in control cells, suggesting that degradation of CaM is impaired in AD lymphoblasts. The rate of CaM degradation was found to be dependent on cellular Ca^{2+} and ROS levels. CaM degradation occurs mainly via the ubiquitin-proteasome system. Increased levels of CaM were associated with overactivation of PI3K/Akt and CaMKII. Our results suggest that increased levels of CaM synergize with serum to overactivate PI3K/Akt in AD cells by direct binding of CaM to the regulatory α-subunit (p85) of PI3K. The systemic failure of CaM degradation, and thus of Ca^{2+}/CaM-dependent signaling pathways, may be important in the etiopathogenesis of AD.

Keywords: Alzheimer's disease, Ca^{2+}/Calmodulin, CaMKII, lymphocytes, PI3K/Akt, ROS.

INTRODUCTION

Dysregulation of calcium homeostasis is among the major cellular alterations in Alzheimer's disease, leading to neuronal dysfunction and ultimately apoptosis [1-3]. As the major cellular Ca^{2+}-binding protein, CaM responds to calcium fluxes by binding and regulating the activity of multiple CaM-dependent proteins [4]. Some of these CaM-binding proteins (CaMBPs) are involved in fundamental events of calcium-mediated neuronal function, as well as in processes such as learning and memory [5]. Moreover, a search for CaMBPs revealed that many of the proteins intimately linked to AD, such as tau or presenilins, may be calmodulin-binding proteins [5]. CaM consists of two homologous domains (N- and C-terminal), and each domain contains two EF-hand Ca^{2+}-binding motifs [6, 7]. Apo-CaM forms a closed conformation by its two homologous domains. Once Ca^{2+} binds to the EF-hand motifs in both domains, the conformation of CaM changes into an open form, allowing for its client proteins to access hydrophobic pockets located in the inner parts of each domain [8, 9]. Consequently, the protein is implicated in a variety of cellular functions, and most importantly CaM levels change with age [10].

Since the concentration of CaM-dependent target proteins exceeds that of cellular CaM, changes in rates of CaM expression and/or degradation will have important physiological effects on the availability of CaM for target protein regulation [11, 12]. In particular, during biological aging, the cellular abundance of CaM is altered, and oxidized fragments accumulate. Underlying these age-dependent changes in CaM abundance may be oxidant-induced structural changes that promote the recognition and degradation of CaM [10].

On the other hand, alterations in calcium signaling are not restricted to neuronal cells. They also have been reported for peripheral cells such as fibroblasts and lymphocytes, which represent easy to obtain diagnostic material [13-15]. Changes in CaM availability and/or in the intrinsic functional properties of the molecule were linked to disruption of Ca^{2+} homeostasis in immortalized lymphocytes from late-onset AD patients [14]. Interestingly, the distinct Ca^{2+} response of AD was associated with enhanced cell proliferation compared to control lymphoblasts from age-matched individuals [16, 17]. These features were considered as peripheral signs of the disease, as current evidence relates the process of neuronal apoptosis occurring in AD to the aberrant reentry of differentiated neurons into the cell cycle [18-20].

The source of signals that drive cell division in the neurons of AD patients is not yet known. Since CaM has long been implicated in the regulation of cell proliferation [21, 22], and alterations in CaM content and activity have been reported in AD brain [23, 24], the possibility that this mole-

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cule could play a significant role in the cell cycle-mediated neurodegeneration in AD should be considered.

We previously reported that Ca^{2+}/CaM stimulates proliferation and increases resistance to serum deprivation-mediated cell death in lymphoblasts from AD patients [16, 17, 25, 26]. It was proposed that there is a functional relationship between Ca^{2+}/CaM and the main signaling pathways controlling cell survival or death depending upon growth factors availability. Moreover, we demonstrated that the survival of peripheral mononuclear cells from AD patients was also dependent on Ca^{2+}/CaM signaling [25]. This observation, together with the fact that similar changes were found in cell signaling molecules or cell cycle regulatory proteins in fresh isolated or EBV-immortalized lymphocytes from AD patients [17], indicates that cell responsiveness is not altered by transformation. The present work aimed at elucidating whether there is a distinct regulation of CaM levels in AD lymphoblasts. CaM is encoded by three genes, *CALM1, 2,* and *3*, which are located on chromosome 2 (2p21.1-p21.3), 14 (14q24–31) and 19 (19q13.2–13.3) [27] respectively. We investigated the expression of these genes and protein levels, as well as determined the rate of CaM degradation in control and AD cells. In addition, we examined crosstalk between CaM and the PI3K/Akt signaling pathway in the stimulation of proliferation of peripheral cells from AD patients.

MATERIAL AND METHODS

Materials

All components for cell culture were obtained from Invitrogen (Carlsbad, CA). Calmidazolium (CMZ), W-13, N-(4-aminobutyl)-5-chloro-1-naphthalene sulfonamide, ionomycin, BAPTA, GSH, trolox and the proteasome inhibitor lactacystin were obtained from Sigma Aldrich (Alcobendas, Spain). The caspase inhibitor benzyloxy-carbonyl-Val-Asp-fluoromethylketone (Z-VAD-Fmk) was obtained from Calbiochem (Darmstad, Germany). Poly (vinylidene) fluoride (PVDF) membranes for western blots were purchased from Bio-Rad (Richmond, CA). Rabbit polyclonal antibodies (pAbs) against human phospho-Akt (Ser473) and rabbit anti-total CaMKII were obtained from Cell Signaling (Beverly, MA) Mouse monoclonal antibody anti-human PI3K p85α (sc-1637) and pAbs such as rabbit anti-human CaM I (FL-149), rabbit anti-human Ub (FL-76), rabbit anti-human pCaMKII (Thr286) (sc-12886-R) and goat anti-human total-Akt (sc-1618) were from Santa Cruz Biotechnologies (Santa Cruz, CA). Rabbit anti-human ß-actin antibody was from Sigma. The enhanced chemiluminiscence (ECL) system was from Amersham (Uppsala, Sweden). All other reagents were of molecular grade.

Cell Lines

A total of 20 patients diagnosed in the department of Neurology of the University Hospital Doce de Octubre (Madrid, Spain) of probable Alzheimer according to NINCDS-ADRDA (National Institute of Neurological and Communicative Diseases and Stroke-Alzheimer's Disease and Related Disorders Association) criteria were used in this study. The average age of onset of the disease was 74±2 years. A group of 20 non-demented age-matched individuals was used as control. The frequency of the ApoE 4 allele was found to be 3% in the control group and 39% in the AD group in agreement with values previously reported for the control and AD population of Spain [28], and consistent with the late-onset form of AD. In all cases peripheral blood samples were obtained after written informed consent of the patients or their relatives.

Establishment of lymphoblastic cell lines was performed in our laboratory as previously described by infecting peripheral blood lymphocytes with the Epstein Barr virus [29]. Cells were grown in suspension in T flasks in an upright position, in approximately 10 ml of RPMI-1640 (Gibco, BRL) medium that contained 2 mM L-glutamine, 100 mg/ml penicillin/streptomycin and, unless otherwise stated, 10 % (v/v) fetal bovine serum (FBS) and maintained in a humidified 5% CO_2 incubator at 37 ºC. Fluid was routinely changed every two days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

Determination of Cell Proliferation

Proliferation was determined by cell counting in a Neubauer chamber. Potential toxicity of the reagents used was routinely checked by trypan blue exclusion under inverted phase-contrast microscopy.

Quantitative Reverse Transcription-PCR

Total RNA was extracted from cell cultures using the Trizol™ reagent (Invitrogen). RNA yields were quantified spectrophotometrically and RNA quality was checked by the A260/A280 ratio and on a 1.2 % agarose gel to observe the integrity of 18S and 28S rRNA. RNA was then treated with DNase I Amplification Grade (Invitrogen). One microgram was reverse transcribed with the Superscript III Reverse Transcriptase kit (Invitrogen). Quantitative real-time PCR was performed in triplicates using TaqMan Universal PCR MasterMix No Amperase UNG (Applied Biosystems) reagent according to the manufacturer's protocol. Primers were used at a final concentration of 20 µM. The sequences of the primers used for real time PCR are listed in Table **1**.

Real time quantitative PCR was performed in the BioRad iQ5 system using a thermal profile of an initial 5-min melting step at 95ºC followed by 40 cycles at 95ºC for 10s and 60ºC for 60s.

Relative mRNA levels of the genes of interest were normalized to ß-actin expression using the simplified comparative threshold cycle delta *CT* method [$2^{-(\Delta CT\ CaM-\ \Delta CT\ Actin}$].

Measurement of Free Intracellular Ca^{2+}

Intracellular Ca^{2+} levels were determined using the Fluo-4-AM probe (Molecular Probes), which binds Ca^{2+} with a 1:1 stoichiometry. Control and AD lymphoblasts were incubated in RPMI medium containing 10 % FBS for 24 h. Then, cells were harvested, washed once with PBS and resuspended in fresh buffer. The cells were then incubated in the dark with 1 µM Fluo-4-AM for 30 min at 37ºC, and the fluorescence was measured at FL-1 (530 nm) in a flow cytometer (EPICS-XL cytofluorimeter (Coulter Científica, Móstoles, Spain)) with an excitation laser at 488 nm. At least 10,000 events per sample were acquired.

Table 1. Sequences of Oligodeoxyribonucleotide Primers Used for Quantitative Real-Time PCR

Gene	Primer Sequence	
	Forward (5' → 3')	Reverse (5'→ 3')
CALM 1	AACAGAAGCTGAATTGCAGGA	AATTCGGGGAAGTCAATGG
CALM 2	ATGGCTGACCAACTGACTGA	CAGTTCCAATTCCTTTGTTG
CALM 3	AACCTTGATCCCCGTGCT	AGGCCTCCTTGAACTCTGC
β-ACTIN	CCAACCGCGAGAAGATGA	CCAGAGGCGTACAGGGATAG

Probes were designed using the Universal ProbeLibrary for Human (Roche Applied Science).

Measurement of Reactive Oxygen Species

The intracellular accumulation of ROS was determined using the fluorescent probe CM-H_2DCFDA (Invitrogen, C6827). After treatment of AD lymphoblasts with the antioxidant agents for 24 hours the cells were collected by centrifugation, resuspended in PBS, and loaded with 10 μM CM-H_2DCFDA during 30 min. Fluorescence measurements were carried out using a POLARstar Galaxy spectrofluorimeter (BMG Labtechnologies, Offenburg, Germany). The excitation wavelength was 495 nm and the emission wavelength was 510 nm.

Immunological Analysis

Cell Extracts

To prepare whole cell extracts, cells were harvested, washed in PBS and then lysed in ice-cold buffer (50 mM Tris pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Nonidet P-40), containing 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate and protease inhibitor Complete Mini Mixture (Roche, Mannhein).

The protein content of the extracts was determined by the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA).

Western Blot Analysis

50-100 μg of protein from whole cell extracts were fractionated on a SDS polyacrylamide gel, and transferred to PVDF membrane (BioRad, Hercules CA). The amount of protein and the integrity of transfer were verified by staining with Ponceau-S solution (Sigma). The filters were then blocked with 1-5 % BSA and incubated, overnight at 4ºC, with primary antibodies at the following dilutions: 1:1000 anti-phospho Akt, 1:1000 anti-Akt, 1:5000 anti-β-actin, 1:500 anti-pCaMKII, 1:1000 anti-CaMKII, 1:500 anti-CaM, 1:500 anti-p85α, 1:500 anti-Ub. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Sigma) and detected with a chemiluminiscent substrate detection system ECL (Amersham). The specificity of the antibodies used in this work, was checked by omitting the primary antibodies in the incubation medium. Blots were stripped and reprobed with anti-ß-actin as a protein loading control. The relative protein levels were determined by scanning the bands with a GS-800 imaging densitometer provided with the Quantity One 4.3.1. software from BioRad, and normalized by that of ß-actin.

Co-Immunoprecipitation Assays

For CaM/Ub experiments, lymphoblasts from control and AD individuals were seeded at an initial density of $1x10^6$ cells/ml and incubated for 6 hours in the absence or in the presence of 1μM MG132. Lysates were collected and 1 mg of protein extract was subjected to immunoprecipitation overnight at 4ºC with anti-CaM antibody. Samples were incubated with protein G Sepharose (GE Healthcare Bio-Sciences) and the resultant immunoprecipitates were washed three times in ice-cold lysis buffer. The samples were then treated with protein sample buffer and boiled prior to immunoblotting. Western blotting was then performed using the indicated antibodies.

For p85/CaM experiments, lymphoblasts from control and AD individuals were seeded at an initial cell density of 1 x 10^6 cells/ml and incubated for 24 h. Protein extracts (1 mg) were subjected to immunoprecipitation overnight at 4ºC with an anti-p85 monoclonal antibody in the presence of 0.1 mM $CaCl_2$ or 2 mM EGTA. Samples were incubated then with protein G Sepharose (GE Healthcare Bio-Sciences) for 2 h at 4ºC. Immunocomplexes were washed three times with ice-cold lysis buffer containing $CaCl_2$, 1 μM CMZ or 2 mM EGTA, suspended with sample buffer, boiled, resolved in SDS-polyacrylamide gel, and transferred onto PVDF transfer membrane filters. Blots were probed with an anti-CaM polyclonal antibody. Membranes were reprobed with the anti-p85 antibody to check for equal immunoprecipitation efficiency.

Statistical Analysis

Unless otherwise stated, all data represent means ± SE. Statistical analysis was performed on the Data Desk package (version 4.0) for Macintosh. Statistical significance was estimated by the Student's t-test or, when appropriated, by analysis of variance (ANOVA) followed by the Fischer's LSD test for multiple comparisons. Differences were considered significant at a level of $p<0.05$.

RESULTS

CaM Levels and Proliferative Activity of Control and AD Lymphoblasts

Fig. (**1A**) shows a comparative analysis of CaM content after 72 h of serum addition between control and AD lymphoblasts. It is shown that lymphoblasts from AD patients had increased levels of CaM compared with CaM content in control cells. In these conditions, lymphoblasts from AD

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patients showed higher rates of cell proliferation Fig. (**1B**) in agreement with previous reports [16]. Treatment of cells with two, structurally unrelated, antagonists of CaM, such as calmidazolium (CMZ) or W13 [30, 31], abrogated the serum-enhanced proliferation of AD cells, without affecting the proliferative activity of control lymphoblasts Fig. (**1B**).

Regulation of CaM Levels

Bearing in mind the apparent relevance of CaM for the enhanced proliferative response of AD lymphoblasts, we were interested in elucidating the mechanisms by which CaM levels are regulated. First, by using real time quantitative PCR, *CALM 1, 2*, and *3* mRNAs expression levels were determined in control and AD lymphoblasts and the results are shown in Table **2**. No differences in CaM mRNA abundance were detected between control and AD cells. Therefore, the increased CaM protein content of AD lymphoblasts, had to be ascribed to a post-transcriptional event.

To test whether the higher CaM content of AD lymphoblasts was dependent on altered protein degradation, we evaluated the stability of the CaM protein, by treating control and AD cells with 20 μg/ml of cycloheximide to inhibit de novo protein synthesis. At the indicated time, steady-state levels of CaM were determined by immunoblotting Fig. (**2**). It is shown that CaM disappeared faster in control than in AD cells. The half-life of CaM was estimated in 22.1 ± 4 h in AD lymphoblasts versus 6.1 ± 1.2 h in control cells. The decreased rates of CaM degradation in AD cells were not due to nonspecific impairment of general protein degradation since no differences were observed in the rate of degradation of β-actin between control and AD lymphoblasts Fig. (**2**).

Table 2. Relative Calmodulin mRNA Abundance

	Control	AD
CALM 1	1 ± 0.109	1.12 ± 0.051
CALM 2	1 ± 0.183	0.86 ± 0,122
CALM 3	1 ± 0.123	1 ± 0.120

Immortalized lymphoblasts from control and AD individuals were seeded at an initial density of 1 x 10^6 x ml^{-1} and cultured for 3 days in RPMI medium containing 10 % FBS. Cells were collected and subjected to RT-qPCR. Relative mRNA levels of the CaM genes were normalized to ß-actin expression, and values for control cells were set as one. Values shown are the mean ± SE for six different cell lines.

Differences in degradation of apo-form of CaM or Ca^{2+}-bound CaM had been reported [10]. For this reason we determined whether half-life of CaM could be influenced by alteration of intracellular Ca^{2+} levels. For these experiments, we incubated control cells with the ionophore, ionomycin, and AD cells with the intracellular Ca^{2+} chelator BAPTA to increase or decrease the respective intracellular Ca^{2+} levels. Fig. (**3**) shows how ionomycin decreased the rate of degradation of CaM in control cells. Conversely, decreasing the intracellular Ca^{2+} content of AD lymphoblasts resulted in enhanced CaM degradation approaching a half-life value close of that of control cells Fig. (**3**). Fig. (**4**) depicts the intracellular Ca^{2+} levels under these conditions. The relative cellular Ca^{2+} content was assessed by Fluo-4AM staining and flow cytometry. As expected, the peak fluorescence was displaced to higher intensity values, indicating increased levels of intracellular Ca^{2+} in control cells treated with ionomycin Fig. (**4**). On the other hand treatment of AD cells with BAPTA effectively decreased the intracellular levels of Ca^{2+}. Fig. (**4**) summarizes the comparison of peak fluorescence

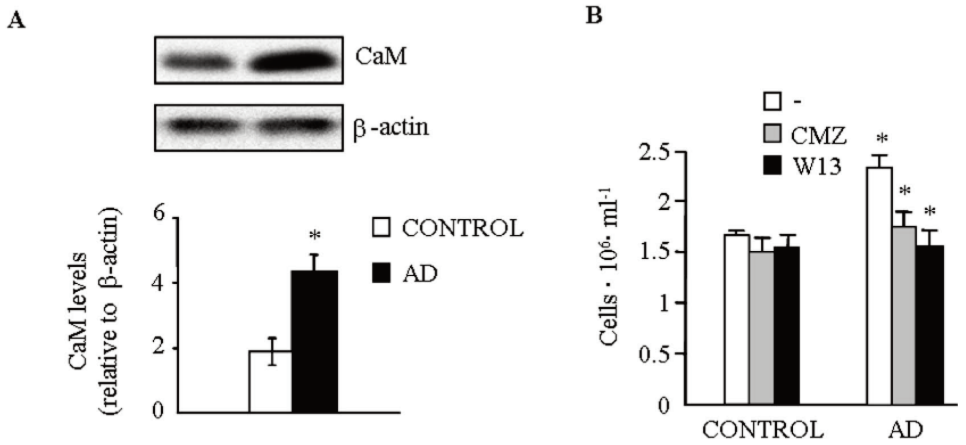

Fig. (1). Calmodulin levels and proliferative activity of control and AD lymphoblasts. **A**: Immortalized lymphoblasts from control and AD individuals were seeded at an initial density of 1 x 10^6 x ml^{-1} and cultured for three days in RPMI medium containing 10 % FBS. CaM was detected by immunoblotting. Band intensity was measured and normalized by that of β-actin. Values shown are the mean±standard (SE) error for 11 independent determinations carried out with cell lines derived from different control and AD individuals. Statistical significance was determined by the t test *p<0.05. **B**: Control and AD lymphoblasts were incubated as above in the absence or in the presence of 1 μM CMZ or 10 μM W-13 for three days. Everyday thereafter, cells were enumerated. Values shown are the mean ± SE for at least six observations carried out with different cell lines from control or AD individuals. Statistical significance was determined by the t test *p<0.05

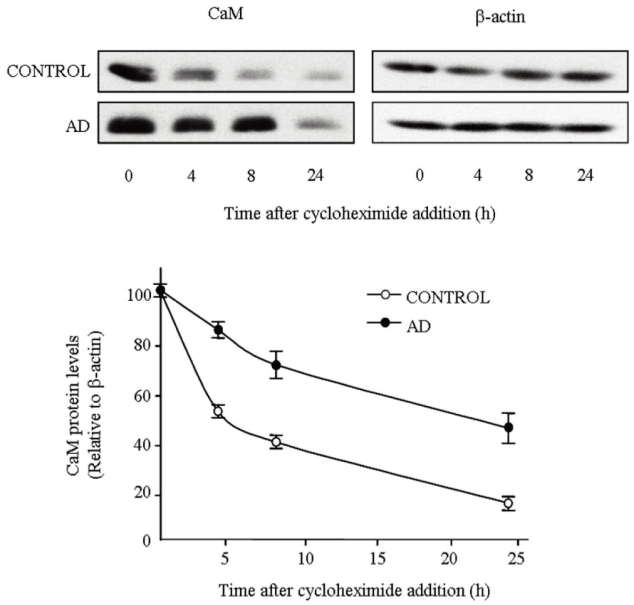

Fig. (2). Half-life of CaM in control and AD lymphoblasts. Cells were serum-deprived and then stimulated by adding 10 % FBS. At this moment cycloheximide (20 μg/ml) was added. Cells were harvested 4, 8, and 24 h thereafter and CaM was detected by immunoblotting. Blots from a representative experiment are shown. The decay of the CaM signal was graphed as a function of time post-cycloheximide addition. Curves were fitted to calculate the half-lives of the proteins, using data from different experiments carried out with of cell lines derived from six control and six AD subjects.

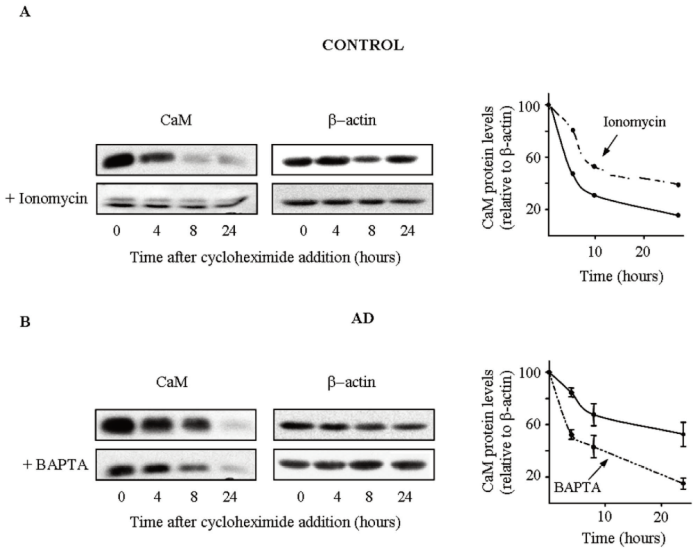

Fig. (3). Influence of intracellular Ca^{2+} levels on the rate of CaM degradation. **A**: Immortalized lymphoblasts from control individuals were seeded at an initial density of 1 x 10^6 x ml^{-1} and serum-deprived for 24 h. Cells were preincubated for 30 minutes in the presence of 1 μM ionomycin and stimulated by adding 10 %FBS. Then, cycloheximide (20 μg/ml) was added. Cells were harvested 4, 8, and 24 h thereafter and CaM was detected by immunoblotting. The experiment was repeated obtaining similar results. **B**: AD lymphoblasts were incubated as above in the presence of the intracellular Ca^{2+} chelator BAPTA at a final concentration of 30 μM. A representative immunoblot is shown. The decay of the CaM signal was graphed as a function of time post-cycloheximide addition. Curves were fitted to calculate the half-lives of the proteins, using data from independent experiments carried out with four cell lines derived from AD individuals.

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displacement in control and AD lymphoblasts in the absence or in the presence of ionomycin or BAPTA. In agreement with previous reports [14], the basal Ca^{2+} concentration of AD lymphoblasts was higher than that of control cells Fig. (**4**).

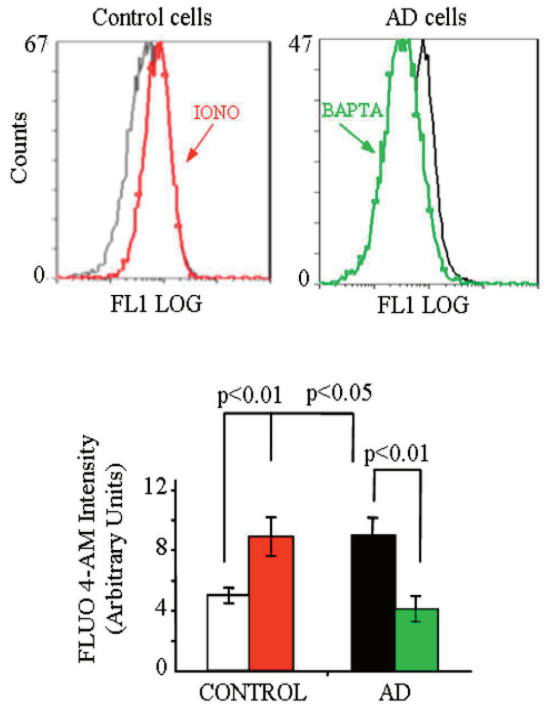

Fig. (4). Intracellular Ca^{2+} levels in control and AD lymphoblasts treated with ionomycin or BAPTA. Control and AD lymphoblasts were incubated with 1 μM ionomycin and 30 μM BAPTA respectively for 24 hours as described in the legend to Fig. (**3**). Intracellular Ca^{2+} levels were then measured with the fluorescent probe Fluo4-AM. The upper plot shows peak displacement to higher or lower fluorescence in the presence of ionomycin or BAPTA respectively. Below, it is shown the relative mean fluorescence intensity ± SE, for at least six observations carried out in cell lines derived from different individuals.

It has been suggested that cellular aging and degenerative diseases lead to increased generation of reactive oxygen species (ROS) and a decline in proteolytic activity, resulting in the progressive accumulation of oxidatively damaged proteins and cellular dysfunction [32]. For this reason, we found it interesting to elucidate whether treatment of AD lymphoblasts with the antioxidants GSH or trolox, modifies the rate of CaM degradation. Fig. (**5A**) shows that both treatments decreased significantly the half-life of CaM in AD lymphoblasts. Fig. (**5B**) shows that untreated AD cells had increased ROS levels than control cells, and the efficacy of either GSH or trolox in decreasing ROS generation up to levels found in control cells.

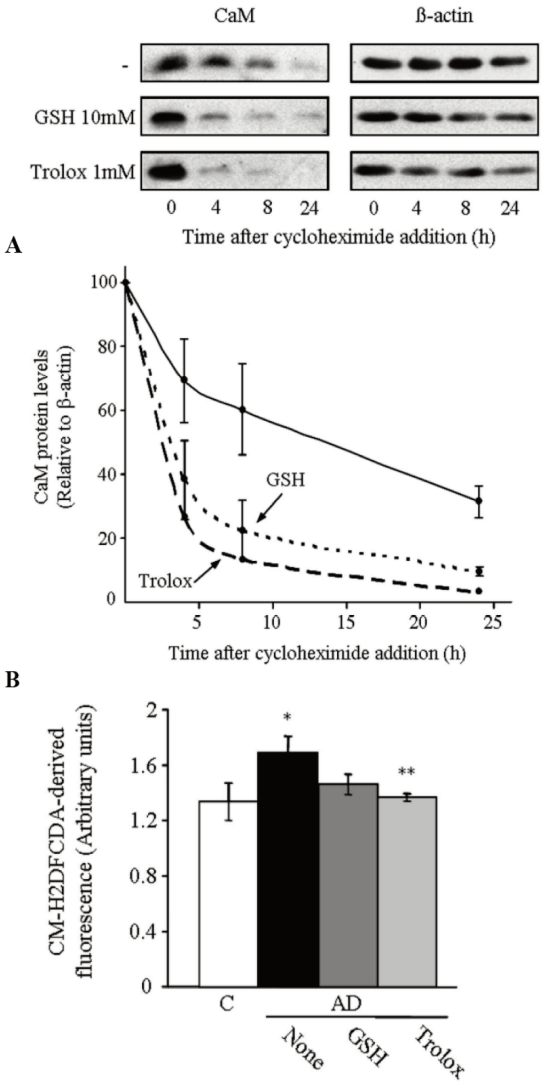

Fig. (5). Effects of antioxidants on the rate of CaM degradation and ROS generation. **A**: Serum-deprived lymphoblasts from AD patients were preincubated for 30 min in the absence or in the presence of 10 mM GSH or 1 mM Trolox, and then stimulated by adding 10%FBS. Afterwards, cycloheximide (20μg/ml) was added. Cells were harvested 4, 8, and 24 h thereafter and CaM was detected by immunoblotting. Results shown are the mean ± SE for at least 3 experiments carried out with different cell lines. **B**: Lymphoblasts from AD patients were incubated for 24 h in the absence or in the presence of 10 mM GSH and 1 mM Trolox. The intracellular ROS levels were determined in control and AD lymphoblasts with the fluoresecent probe CM-H_2DFCDA. Values shown are the mean ± SE for independent determinations carried out with eight different cell lines derived from control or AD individuals. *p<0.05 significantly different from control cells. **p<0.05 significantly different from AD cells without antioxidant treatment.

The Degradation of the CaM Protein is Mainly Proteasome-Dependent

To elucidate the involvement of the major pathways for protein turnover in CaM degradation, we used a battery of protease inhibitors with selective specificity. Data in Fig. (**6A**) show that both in control and AD lymphoblasts, CaM accumulated only in the presence of the highly selective proteasome inhibitor lactacystin. In contrast, treatment of cells with the lysomotropic agents hydroxychloroquine, CH_3NH_2 or NH_4Cl had no effect on cellular CaM content Fig. (**6A**). Similarly, the lack of effect of z-VAD-fmk ruled out the involvement of caspases on CaM degradation Fig. (**6A**). None of these inhibitors increased the levels of ß-actin, which was used as control. Collectively, these results suggested a predominant role for the proteasome system on CaM proteolysis.

To investigate if CaM degradation was accompanied by ubiquinitation of the molecule, we incubated control and AD lymphoblasts in the absence or in the presence of the proteasome inhibitor MG132 to ensure that ubiquitinated CaM was accumulated. CaM was imnunoprecipitated from these cells extracts with the anti-CaM antibody and then probed for ubiquitin with the anti-Ub antibody Fig. (**6B**). It is shown that indeed CaM is ubiquitinated. Although small, it seems to be a decrease in CAM polyubiquitination in AD cells Fig. (**6B**).

Ca^{2+}/CaM Regulation of AD Lymphoblasts Activity

The upregulation of CaM protein levels in lymphoblasts from AD patients raises the question as to whether higher CaM levels are related with increased activity of Ca^{2+}/CaM targets in these cells lines. Fig. (**7A**) shows, that indeed CaMKII was overactivated in AD cells as compared to control cells, and that the CaM antagonist CMZ prevented CaMKII autophosphorylation. Similarly, PI3K/Akt activity, monitored by Akt phosphorylation, is enhanced in AD lymphoblasts Fig. (**7B**). The overactivation was prevented by the CaM antagonists CMZ and W-13, but not with the CaMKII inhibitor KN-62, indicating that CaM-induced activation of PI3K/Akt was not mediated by this CaM-dependent kinase. These inhibitors didn't change basal levels of PI3K activity in control cells, suggesting the existence a threshold for CaM activation. Since the p85 regulatory subunit of PI3K/Akt had been shown to bind CaM [33, 34], we investigated whether the enhanced PI3K/Akt activity in AD lymphoblasts was due to differences in the CaM-p85 interaction between control and AD cells. To this end, cell lysates from control and AD lymphoblasts were immunoprecipitated with the anti-p85 antibody. The immunoprecipitates were resolved by SDS-PAGE, and the immunoblots were probed with an anti-CaM antibody. Clearly, CaM was found to co-immunoprecipitate with p85 in AD extracts Fig. (**8**). Immunocomplexes formation was scarce in control extracts Fig. (**8**). As previously described [34], CaM and p85 interaction was found to be Ca^{2+}-dependent Fig. (**8**). When CMZ or W-13 was added during the immunoprecipitation process the association was strongly reduced Fig. (**8**). These results suggest that treatment of AD cells with CaM antagonists could prevent CaM-mediated overactivation of PI3K, and therefore overcome the enhanced proliferative activity of AD lymphoblasts [17].

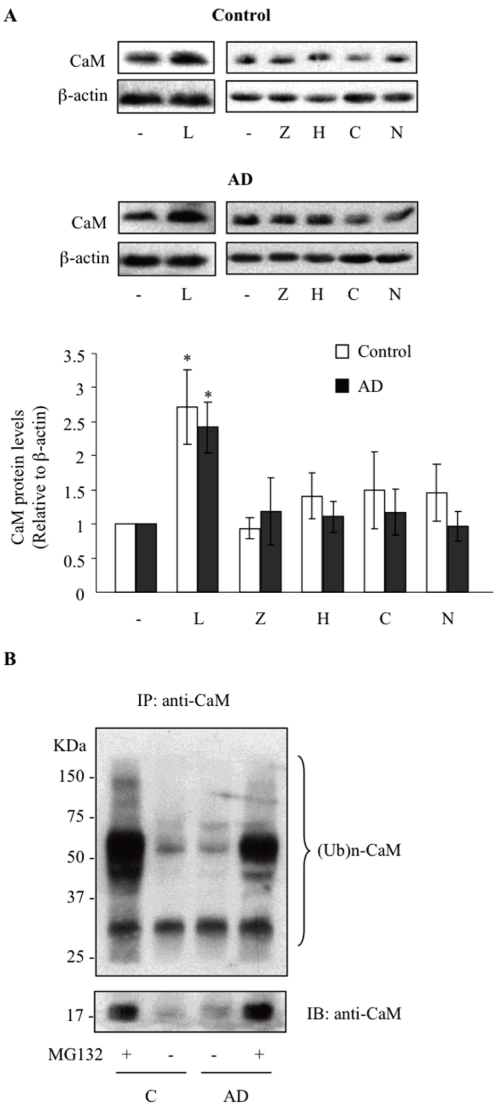

Fig. (6). CaM degradation appears to occur in the proteasome. **A**: Control and AD lymphoblasts were incubated for 24 h in the presence of the proteasome inhibitor lactacystin (L) (15 μM), the caspase inhibitor z-VAD-fmk (Z) (50 μM) or the autophagy inhibitors, hydroxychloroquine (H) (250 μM); CH_3NH_2 (C) (15 mM) or NH_4Cl (N) (15 mM). CaM accumulated only in the presence of lactacystin. Values shown are the mean ± SE for 6-8 determinations carried out with cell lines derived from different individuals. **B**: Control and AD lymphoblasts were incubated for 6 h in the absence or in the presence of the proteasome inhibitor MG132 (1 μM). Lysates from these cell cultures were immunoprecipitated with anti-CaM antibody and probed for ubiquitin with the anti-Ub antibody. Efficiency of the immunoprecipitation procedure was checked by reprobing the membranes using the anti-CaM antibody The experiment was repeated once obtaining similar results.

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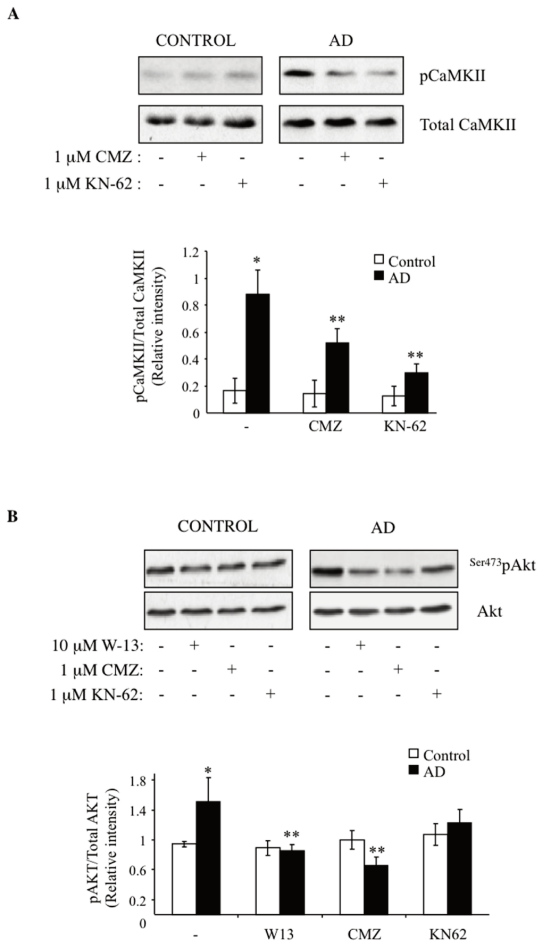

Fig. (7). CaMKII and PI3K/Akt activities in control and AD lymphoblasts. **A**: Immortalized lymphoblasts from control and AD patients were seeded at an initial density of 1 x 10^6 x ml^{-1} and cultured for 24 h in the absence or in the presence of the CaM antagonists CMZ (1 μM) or the CaMKII inhibitor KN-62 (1 μM). Whole cell lysates were immunoblotted with antibodies anti-phospho-CaMKII (Ser286) and total CaMKII. The densitometric data represents the mean ± SE for three independent determinations carried out with different cell lines *p<0.05 significantly different from control cells. **p<0.05 significantly different from AD cells incubated without inhibitors. **B**: Control and AD lymphoblasts were incubated as above in the absence or in the presence 1 μM CMZ, 10 μM W-13 or 1 μM KN-62. Phospho-Akt (Ser473) and total Akt were determined by immunoblotting. Representative immunoblots are presented. The densitometric data below represents the mean ± SE for determinations carried out with six different control and AD cell lines. *p<0.01 significantly different from control cells. **p<0.01 significantly different from AD cells incubated without inhibitors.

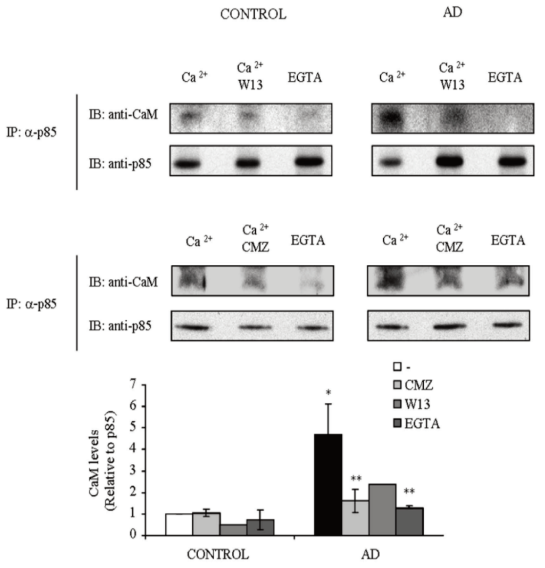

Fig. (8). Effects of Ca^{2+} levels and calmodulin antagonists on CaM binding to p85 regulatory subunit of PI3K. Lysates from control and AD lymphoblasts were immunoprecipitated with the anti-p85 antibody (α-p85) in the presence of 0.1 mM $CaCl_2$ or 2 mM EGTA, and 0.1 mM $CaCl_2$ plus 1 μM CMZ or 10 μM W-13. Immunocomplexes were analyzed by Western blot with an anti-CaM antibody. Efficiency of p85 immunoprecipitation, in the different conditions, was checked by reprobing the membranes with the anti-α-p85 antibody. Representative experiments are shown. W-13 was used in two different experiments, while the effect of CMZ and EGTA was determined in four different immunoprecipitation experiments. Below it is presented the densitometric analysis. When present the bars represent the SE of the mean. *p<0.01, significantly different from control cells, **p<0.01 significantly different from AD cells incubated without inhibitors.

DISCUSSION

Previous work from our laboratory indicated impaired Ca^{2+}/CaM-dependent signaling in immortalized lymphocytes from AD patients, resulting in increased proliferative activity upon serum stimulation, and higher resistance to serum deprivation-induced apoptosis [16, 17, 25]. The present work was undertaken to evaluate whether the regulation of CaM content was altered in lymphoblastoid cell lines from late-onset AD patients. These cell lines, easily accessible, had previously proved to be useful model to study cell cycle-related events associated to neurodegeneration [16, 35]. Three major conclusions can be drawn from our work: first, CaM levels are increased in AD lymphoblasts; second, CaM half-life is enhanced in AD cells, and third, the rate of CaM degradation is dependent on intracellular Ca^{2+} levels and ROS status of the cell.

Increased levels of CaM were associated with overactivation of PI3K/Akt and enhanced proliferation of AD cells. These results are in line with the known role of CaM in regulating cell cycle progression [22]. In addition, both processes were sensitive to CaM antagonists, although these com-

pounds were not able to decrease proliferation or PI3K/Akt activity in control cells [17], indicating the existence of a threshold of CaM dependent activation of PI3K/Akt and cell proliferation.

Our results suggested that the increased CaM levels in AD cells synergize with serum to overactivate PI3K/Akt pathway. In agreement with previous reports [33, 34] we found that CaM is able to bind to the 85 KDa regulatory subunit of PI3K (p85). Moreover it was observed a significant higher binding of CaM to p85 in AD lymphoblasts compared to control cells, thereby resulting in enhanced Akt phosphorylation.

The up-regulation of CaM levels in AD lymphoblasts is not the consequence of altered expression of any of the three different genes that encode CaM, but rather the result of decreased rates of CaM degradation. The half-life of CaM in AD lymphoblasts was estimated in 22 h, approximately 3 times fold of that of control cells. These values are in consonance with the reported half-life of CaM in rat brain [36]. It is worth to highlight that CaM has a very much shorter half-life than other calcium homeostasis related enzymes, such as plasma membrane Ca-ATPase (12 days) in the same tissue, but resembles the half-life of the bulk of cell proteins [36].

CaM exhibited proteasome-sensitive turnover. CaM accumulated when control or AD cells were treated with lactacystin, while CaM content was not affected by either caspase or autophagy inhibitors. The proteasomal degradation of CaM was accompanied by ubiquitination of the molecule, as co-immunoprecipitation of ubiquitin and CaM was observed in cell extracts from control and AD lymphoblasts. Apparently there is a slight decrease in the polyubiquitation of CaM in AD cells, which may contribute to the reduced CAM degradation in these cell lines. However, it was reported that aged or oxidized CaM can be efficiently degraded by either the 20S or 26S proteasome in an ubiquitin-independent manner [10, 37]. More likely, both ubiquitin-dependent and independent mechanisms of proteasome degradation of CaM can operate *in vivo*. Further work is needed to clarify this point and to evaluate their potential contribution to CaM levels regulation.

Work from our and other laboratories demonstrated no global proteasome activity deficiency in lymphocytes from AD patients [17, 38, 39]. Indeed we reported enhanced proteasome-dependent degradation of the CDK inhibitor p27 in AD lymphoblasts [17]. On the other hand, the rate of β-actin degradation is similar in both control and AD lymphoblasts. Thus, the decreased rate of CaM degradation cannot be the result of nonspecific impairment of protein degradation in lymphoblasts from AD patients.

Intracellular Ca^{2+} levels and oxygen reactive species content appear to regulate the rate of CaM degradation in immortalized lymphocytes. Buffering the intracellular Ca^{2+} increase by BAPTA, restored the normal rate of CaM degradation. Conversely, treatment of control cells with ionomycin increased the CaM half-life in control cells. Reducing the rate of CaM degradation could be the cellular response to buffer Ca^{2+} overload. Previous work indicated that ubiquitination and degradation of CaM *in vitro* show opposite sensitivity to Ca^{2+} [10]. The rate of CaM degradation decreased in the presence of Ca^{2+}. It was suggested that ubiquitinated CaM could retain sufficient Ca^{2+} binding capacity to maintain a structure too rigid to be unfolded and directed to the proteasome [10]. On the other hand, treatment with antioxidants also normalized CaM degradation in AD lymphoblasts. It is known that enhanced ROS generation perturbs Ca^{2+} fluxes [40]. Thus, the effect of ROS controlling the rate of CaM degradation may play an additional role in ROS-induced disruption of Ca^{2+} homeostasis.

Despite the increased levels of CaM, AD lymphoblasts showed elevated intracellular concentration of Ca^{2+} in agreement with reports showing diminished Ca^{2+} buffering capacity in lymphoblasts from late-onset AD patients [14]. Moreover, decreased Ca^{2+} binding proteins in AD brain had also been reported [41]. These observations together with altered Ca^{2+} fluxes may contribute to the increased cytosolic Ca^{2+} of AD cells [42].

The binding of up to four calcium ions to CaM elicits significant conformational changes that increase the exposure of hydrophobic residues, e.g., Met, that serve as binding sites for target proteins [4]. In addition, the extent of Ca^{2+} saturation and location of bound Ca^{2+} ions within the CaM molecule also influence CaM's binding to targets [43]. As already mentioned we observed enhanced binding of CaM with the p85 regulatory subunit of PI3K in AD lymphoblasts. The activity of PI3K, as assessed by monitoring Akt phosphorylation, was also enhanced in AD lymphoblasts and sensitive to CaM antagonists. The activity of other CaM-dependent protein, CAMKII, was also found to increase in lymphoblasts from AD patients. This protein plays an important role in controlling the cellular response to serum-deprivation-induced apoptosis in immortalized lymphocytes [25, 26].

Despite the role of Ca^{2+}/CaM signaling in AD pathology, the mechanisms controlling CaM levels have received little attention. The increased CaM content of AD lymphoblasts reported here contrasts with previously reported reduction of CaM in AD-affected cerebral cortex [44]. The discrepancy may be due to differences in the ability of some anti-CAM antibodies to recognize CaM only in certain conformational states. Overactivation of PI3K/Akt has been, however, reported in AD brain. Increased posphorylation of Akt was detected in AD brain [45]. Furthermore, overactivated Akt in AD brain is accompanied by increased phosphoryaltion of Akt substrates, such as GSK3 or mTOR, and decreased levels of the CDKi p27 [46]. In this regard it is worth mentioning the enhanced Ca^{2+}/CaM/PI3K/Akt-dependent degradation of p27 previously reported in AD lymphoblasts [17]. Similarly, impaired CaMKII activation has been also detected in AD brain, associated with increased phosphorylation of tau and neurofibrillary tangle formation [47]. Thus, our results obtained in peripheral, easily accessible cells from AD patients, suggest that CaM degradation may also be perturbed in AD brain. Altered CaM levels in AD brain could play a role in the cell cycle disturbances-induced neuronal apoptosis. Therefore, the systemic failure of mechanism involved in CaM degradation, and thus of Ca^{2+}/CaM-dependent signaling pathways may be important to unravel the pathomechanism of AD.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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La inactivación de ERK ½ por CaMKII regula los niveles de p21 y la supervivencia en linfocitos inmortalizados de pacientes de alzhéimer

4

Artículo #3 : **Noemí Esteras**, Carolina Alquézar, Félix Bermejo-Pareja, Emilia Bialopiotrowicz, Urszula Wojda, Ángeles Martín-Requero (2012). "Downregulation of ERK1/2 activity by CaMKII modulates p21 and survival of immortalized lymphocytes from Alzheimer's disease patients". Submitted.

Resumen

Con anterioridad, habíamos descrito que la mayor actividad de CaM/CaMKII de los linfoblastos de EA protege a estas células de la muerte inducida por la privación de suero. Este hecho se acompaña de una disminución acusada de la activación de la vía de las MAPK (ERK1/2) y un aumento en los niveles de p21. El objetivo de este trabajo ha sido investigar los mecanismos moleculares que están detrás de la regulación diferencial de p21 en las células de los pacientes de EA tras la retirada de suero. El análisis de la vida media de la proteína permite descartar que tenga lugar una alteración en la degradación de la misma. Sin embargo, el análisis por qRT-PCR muestra un aumento en los niveles de ARN mensajero de p21 tras la retirada de suero. El inhibidor de ERK1/2, PD98059, previene la muerte celular inducida por la privación de suero en los linfoblastos controles e induce un aumento en los niveles del mensajero y de proteína de p21. Por su parte, el inhibidor de PI3K/Akt, Ly29004, tiene escasa influencia en estos procesos. El antagonista de CaM, CMZ, y el inhibidor de CaMKII, KN-62, revierten la mayor supervivencia mostrada por las células de EA y reducen los niveles de p21 al aumentar la actividad de ERK1/2. La regulación transcripcional de p21 en estas células no parece depender de p53, sino que nuestros resultados indican que ERK1/2 regula la expresión de p21 a través del factor de transcripción FOXO3a. Aparentemente, la menor actividad de ERK1/2 de linfoblastos de EA favorece la localización nuclear de FOXO3a, al impedir, al menos parcialmente, la fosforilación y la exportación del factor de transcripción al citosol para ser degradado por un mecanismo que parece estar mediado por MDM2. La retención de FOXO3a en el núcleo permite una mayor transcripción de p21. Por otra parte, observamos un aumento en el contenido de p21 en el citoplasma de los linfoblastos de EA que podría explicar los efectos antiapoptóticos de esta proteína en células derivadas de pacientes de EA.

Downregulation of ERK1/2 activity by CaMKII modulates $p21^{Cip1}$ levels and survival of immortalized lymphocytes from Alzheimer's disease patients.

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Abstract: Previously, we reported a Ca^{2+}/calmodulin (CaM)-dependent impairment of apoptosis induced by serum deprivation in Alzheimer's disease (AD) lymphoblasts. These cell lines showed downregulation of ERK1/2 activity and elevated content of p21 compared to control cells. The aim of this study was to delineate the molecular mechanism underlying the distinct regulation of p21 content in AD cells. Quantitative RT-PCR analysis demonstrated increased p21 mRNA levels in AD cells. The ERK1/2 inhibitor, PD98059, prevented death of control cells and enhanced p21 mRNA and protein levels. The CaM antagonist, CMZ, and the CaMKII inhibitor, KN-62, normalized the survival pattern of AD lymphoblasts by augmenting ERK1/2 activation and reducing p21 mRNA and protein levels. Upregulation of p21 transcription in AD cells appears to be the consequence of increased activity of FOXO3a as the result of diminished ERK1/2-mediated phosphorylation of this transcription factor, which in turn facilitates its nuclear accumulation. MDM2 protein levels were decreased in AD cells relative to control lymphoblasts, suggesting an impairment of FOXO3a degradation.

Key words: lymphocytes, Alzheimer's disease, p21, apoptosis, calmodulin, CaMKII, ERK1/2, FOXO3a

1. Introduction

Alzheimer's disease (AD) is a late-onset human neurodegenerative disorder marked by a progressive dementia and a spectrum of behavioral alterations. Whereas the hallmarks of AD, neurofibrillary tangles and amyloid-β containing plaques are well established, the cause of neuronal loss remains largely elusive. Cumulative evidence has associated the aberrant re-expression of some cell cycle regulatory proteins with neuron vulnerability and neurodegeneration in AD (McShea, et al., 2007, Webber, et al., 2005). Cell cycle re-entry appears to represent an early and critical event in AD, leading to the development of AD-related pathology such as hyperphosphorylation of tau and amyloid-β deposition and ultimately inducing neuronal cell death (Bonda, et al., 2010, Lee, et al., 2009). It is believed that certain neurons are able to reactivate cell-cycle activity in response to different triggers of neuronal apoptosis, including the withdrawal of growth factors (Park, et al., 1998) and other detrimental factors (Kruman, et al., 2002, Verdaguer, et al., 2002, Zhu, et al., 2007). Abnormal cell cycle re-entry leads to neuronal dead, however, in AD, the activation of neuronal cell cycle may result in apoptosis avoidance (Jellinger, 2006, Raina, et al., 2000), allowing the cells to arrest in G2, accumulating oxidative damage which in turn would induce its death according to the two-hit hypothesis (Moh, et al., 2011, Zhu, et al., 2004).

Cell cycle dysregulation is not restricted to neurons, since peripheral cells from AD patients such as lymphocytes or fibroblasts have been shown to display cell cycle-related alterations (Bialopiotrowicz, et al., 2011, de las Cuevas, et al., 2003, Nagy, et al., 2002, Stieler, et al., 2012, Tatebayashi, et al., 1995, Zhang, et al., 2003). It appears therefore that cell cycle disturbances represent a systemic aspect of the disease. While very limited data exist on the contribution of aberrant cell cycle in lymphocytes to the clinical phenotype of AD, future research should address this question. There is no doubt that peripheral cells from patients provide convenient material to study cell cycle-related events associated with neurodegeneration.

Previous work from this laboratory indicated that immortalized lymphocytes from AD subjects show enhanced proliferative activity (de las Cuevas, et al., 2003, Munoz, et al., 2008) and

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more resistance to serum withdrawal-induced apoptosis in a Ca^{2+}/CaM-dependent manner (Bartolome, et al., 2007, de las Cuevas, et al., 2005). It was also shown that both CaM content and activity were enhanced in AD lymphoblasts (Esteras, et al., 2012). Two cell cycle regulatory proteins, the CDK inhibitors $p27^{Kip1}$ and $p21^{Cip1}$, from now on p27 and p21, are* ultimately responsible for the enhanced proliferation and increased resistance to cell death, respectively. Whereas downregulation of p27 induces the enhanced proliferative response of immortalized lymphocytes from AD patients (Munoz, et al., 2008), upregulation of p21 seems to help AD cells to escape from serum deprivation-induced apoptosis (Bartolome, et al., 2010).

A number of recent studies pointed out that in addition to being an inhibitor of cell proliferation, p21 may protect cells from apoptosis (Gartel, 2009). For example, it has been reported that upregulation of p21 blocked the oxidative stress-induced death of human myeloma U266 cells (Kim, et al., 2001) and rendered resistance to chemotherapy drugs in other types of cancer cells (Gareau, et al., 2011). Thus the increase in p21 cellular content in AD lymphoblasts may confer these cells a survival advantage.

This work was undertaken to unravel the molecular mechanisms underlying the Ca^{2+}/CaM-mediated resistance of AD lymphoblasts to cell death induced by serum deprivation and the role of Ca^{2+}/CaM in the regulation of p21 cellular levels. We tested the hypothesis that enhanced Ca^{2+}/CaM signaling would protect AD cells from apoptosis via upregulation of p21. Our data suggest that CaMKII indirectly regulates the FOXO3a-mediated activation of p21 transcription by preventing its phosphorylation mediated by ERK1/2 and subsequent translocation and degradation of this transcription factor via an MDM2-mediated ubiquitin-proteasome pathway.

2. Methods

2.1. Materials.

All components for cell culture were obtained from Invitrogen (Carlsbad, CA). Calmidazolium (CMZ), KN62 and pifithrin-α (PFT-α) were obtained from Sigma Aldrich (Alcobendas, Spain). LY294002 and PD98059 were obtained from Calbiochem (Darmstadt, Germany). PVDF (polyvinylidene difluoride) membranes for Western blots were purchased from Bio-Rad (Richmond, CA). Rabbit anti-human polyclonal antibodies, such as FOXO3a, ERK1/2, CaMKII, p53 and phospho-p53 (Ser 15) and rabbit anti-human monoclonal antibodies such as p21 and phospho-ERK1/2 were from Cell Signaling. Rabbit anti-human phospho-CaMKII (Thr286) (sc-12886-R), and mouse anti-human α-tubulin (sc-23948) were from Santa Cruz Biotechnologies (Santa Cruz, CA). Rabbit anti-human β-actin antibody was from Sigma. Rabbit anti-human MDM2 was from Millipore (Darmstadt, Germany). Mouse anti-human Lamin B was from Calbiochem. The enhanced chemiluminiscence (ECL) system was from Amersham (Uppsala, Sweden). All other reagents were of molecular grade.

2.2. Cell lines

A total of 34 patients diagnosed in the department of Neurology of the University Hospital Doce de Octubre (Madrid, Spain) of probable Alzheimer according to NINCDS-ADRDA (National Institute of Neurological and Communicative Diseases and Stroke-Alzheimer's Disease and Related Disorders Association) criteria were used in this study. Of the 34 patients, 20 had mild AD (DSM-III-R, Mini Mental State Examination (MMSE) score between 18-24), 7 had moderate AD (MMSE: 10-18), and 7 had severe AD (MMSE: <10). A group of 23 non-demented age-matched individuals was used as control. In all cases peripheral blood samples were obtained after written informed consent of the patients or their relatives. A summary of demographic characteristics of all subjects enrolled in this study is reported in Table 1.

Establishment of lymphoblastic cell lines was performed in our laboratory as previously described by infecting peripheral blood lymphocytes with the Epstein-Barr virus (Koistinen, 1987). Cells were grown in suspension in T flasks in an upright position, in approximately 8 mL of RPMI-1640 (Gibco, BRL) medium that contained 2 mM L-glutamine, 100 μg/mL penicillin/streptomycin and, unless otherwise stated, 10 % (v/v) fetal bovine serum (FBS) and maintained in a humidified 5% CO_2 incubator at 37 ºC. Fluid was routinely changed every two days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

2.3. Cell survival assay.

The cell suspension was mixed with a 0.4% (w/v) Trypan Blue solution (Sigma), and the number of live cells was determined using a hemocytometer. Cells failing to exclude the dye were considered nonviable. In addition, apoptosis was characterized by chromatin condensation/fragmentation, as determined by cell fixation followed by DAPI staining and fluorescence microscopy examination.

2.4. Preparation of whole-cell extracts and subcellular fractionation.

To prepare whole-cell extracts, cells were harvested, washed in PBS and then lysed in ice-cold buffer (50 mM Tris pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Nonidet P-40), containing 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate and protease inhibitor Complete Mini Mixture (Roche, Mannhein).

To separate the cytosolic and nuclear fractions, cells were harvested, washed in PBS and then lysed in ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM ethylendiaminetetraacetic acid (EDTA), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM PMSF and protease inhibitor mixture. After extraction on ice for 15 minutes, 0.5% Nonidet P-40 was added and the lysed cells were centrifuged at 4000 rpm for 10 minutes. Supernatants containing cytosolic proteins were separated, and nuclei were washed twice with the hypotonic buffer, and then lysed in hypertonic buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM ethylendiaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM PMSF and protease inhibitor mixture. After extraction on ice for 30 minutes, the samples were centrifuged at 15000 rpm for 15 minutes at 4 ºC. Antibodies to α-tubulin and to Lamin B were used to assess the purity of the fractions.

The protein content of the extracts was determined by the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA).

2.5. Western Blot Analyses

50-100 μg of protein from cell extracts were fractionated on a SDS polyacrylamide gel, and transferred to a PVDF membrane which was then blocked with 1-5% bovine serum albumin (BSA) or 5% nonfat milk, and incubated overnight at 4ºC, with primary antibodies at the following dilutions: 1:1000 anti-p21, 1:1000 anti-FOXO3a, 1:2000 anti-phospho ERK1/2, 1:1000 anti-ERK1/2, 1:500 anti-pCaMKII, 1:1000 anti-CaMKII, 1:5000 anti-β-actin, 1:1000 anti-α-tubulin, 1:1000 anti-Lamin B, 1:1000 anti-phospho p53 (Ser 15), 1:1000 anti-p53 and 1:1000 anti-MDM2. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Bio-Rad) and detected with a chemiluminiscent substrate detection system ECL (Amersham). The specificity of the antibodies used in this work was checked by omitting the primary antibodies in the incubation medium. Protein band densities were quantified using Image J software (NIH, Bethesda, Maryland, USA) after scanning the images with a GS-800 densitometer from BioRad.

2.6. Quantitative Real-Time PCR.

Total RNA was extracted from cell cultures using Trizol™ reagent (Invitrogen). RNA yields were quantified spectrophotometrically and RNA quality was checked by the A260/A280 ratio and on a 1.2% agarose gel to observe the integrity of 18S and 28S rRNA. RNA was then treated with DNase I Amplification Grade (Invitrogen). One microgram was reverse transcribed with the Superscript III Reverse Transcriptase kit (Invitrogen). Quantitative real-time PCR was performed in triplicates using TaqMan Universal PCR MasterMix No Amperase UNG (Applied Biosystems) reagent according to the manufacturer's protocol. Primers were designed using the Universal ProbeLibrary for Human (Roche Applied Science) and used at a final concentration of 20 μM. The sequences of the forward and reverse primers used are the following: for p21 5'-cgaagtcagttccttgtggag-3' and 5'-catgggttctgacggacat-3'; for β-actin 5'-ccaaccgcgagaagatga-3' and 5'-ccagaggcgtacagggatag-3'.

Real time quantitative PCR was performed in the BioRad iQ5 system using a thermal profile of an initial 5-min melting step at 95ºC followed by 40 cycles at 95ºC for 10s and 60ºC for 60s.

Relative mRNA levels of the genes of interest were normalized to β-actin expression using the simplified comparative threshold cycle delta *CT* method [$2^{-(\Delta CT\,p21-\,\Delta CT\,Actin}$].

2.7. Confocal laser scanning microscopy

Immortalized lymphocytes from control and AD individuals were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and incubated in serum-free RPMI medium for 24 hours. Cells were fixed for 15 minutes in 4% paraformaldehyde in PBS and blocked and permeabilized with 0.5% Triton X-100 in PBS-0.5% BSA for 20 minutes at room temperature. Then, cells were incubated with rabbit anti-p21 monoclonal antibody (Cell Signaling). After washing with PBS, cells were incubated with Alexa Fluor 488-conjugated anti-rabbit antibody. For nuclear staining, samples were incubated with DAPI (Sigma) at 4 mg/mL in PBS for 15 minutes at room temperature. The preparations were mounted on FluorSave reagent (Calbiochem, Madrid, Spain) and visualized with the Leica TCS-SP2-AOBS confocal microscope system (Heidelberg, Germany).

2.8. Statistics

Statistical analyses were performed on GraphPad Prism 5 for Macintosh (La Jolla, CA, USA). All

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the statistical data are presented as mean ± standard error of the mean (SEM). Statistical significance was calculated using an unpaired, two-tailed Student's *t*-test or, when appropriated, by analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* testing. A value of $p<0.05$ was considered significant.

3. Results

3.1. CaM and ERK1/2 control of cell survival and p21 content.

Data in Fig. 1 confirm and extend our previous findings that lymphoblasts from AD patients show increased resistance to cell death induced by serum deprivation in a Ca^{2+}/CaM and ERK1/2 activity dependent manner (Bartolome, et al., 2007, Bartolome, et al., 2010). The CaM antagonist, calmidazolium (CMZ) as well as the CaMKII inhibitor KN-62, rescued the normal cell response to serum deprivation, inducing apoptosis in AD cells, suggesting the involvement of CaMKII in the regulation of cell survival. The fact that these drugs didn't affect the survival of control cells suggests a threshold for CaM content/activity as the survival signal. On the other hand, the inhibitor of ERK1/2, PD98059, prevented the serum deprivation-induced apoptosis in control cells without significantly changing survival of AD lymphoblasts (Fig. 1B). To examine the apoptotic status of control and AD lymphoblasts after serum deprivation, cells were observed in a fluorescence microscopy following DAPI staining, which specifically binds to DNA. As shown in Fig. 1, the addition of CMZ or KN-62 to AD lymphoblasts induced chromatin condensation in some nuclei while PD98059 prevented the serum deprivation-induced apoptosis in control cells (Fig. 1C). Taken together, these observations suggest a crosstalk between CaMKII and ERK1/2 in regulating the cellular response to serum deprivation. An inverse relationship was found between CaMKII and ERK1/2 activities, as well as between ERK1/2 phosphorylation status and p21 levels in human lymphoblasts. As shown in Fig. 1D, AD lymphoblasts displayed higher activity of CaMKII, as monitored by the enhanced autophosphorylation, together with decreased ERK1/2 activity, when compared with control cells. In parallel, levels of p21 increased in agreement with previous reports (Bartolome, et al., 2010). Inhibition of CaMKII with CMZ treatment in AD cells restored the activity of ERK1/2 and diminished the p21 cellular content up to the levels observed in control cells. Under these conditions, AD cells underwent apoptosis as control cells did. Thus, it appears that CaM/CAMKII/ERK1/2 signaling pathway controls cell survival under trophic factor deprivation by regulating p21 levels.

3.2. Regulation of p21 levels

To elucidate the molecular basis for the upregulation of p21 in AD lymphoblasts, we tested whether this effect was dependent on altered protein degradation. To this end, we evaluated the stability of the p21 protein after serum-deprivation, by treating control and AD cells with 20 μg/mL of cycloheximide to inhibit *de novo* protein synthesis. At the indicated time, steady-state levels of p21 protein were determined by immunoblotting (Fig. 2). As in other cell types, p21 is a short-lived protein in human lymphoblasts. Inhibition of protein synthesis with cycloheximide led to a rapid decrease in p21 levels, with less than 50% remaining after 2 hours. It is shown that p21 half-life is identical in both control and AD lymphoblasts. We next determined, by quantitative RT-PCR, p21 mRNA levels. As shown in Fig. 3, lymphoblasts from AD patients show an increase in expression of p21 mRNA as compared with control cells, in consonance with the different content of this protein in control and AD lymphoblasts. Inhibition of CaMKII by CMZ or KN-62 abrogated the increased transcription of p21 in AD cells, while inhibiting the PI3K/Akt pathway by Ly294002 had no consequences in p21 mRNA levels (Fig. 3). On the contrary, the ERK1/2 inhibitor PD98059 was able to increase p21 mRNA levels in control cells without affecting the transcription of the gene in AD lymphoblasts. Under these conditions, and in agreement with previous reports (Bartolome, et al., 2010), control cells were found to be resistant to cell death induced by serum deprivation (Fig. 1C). Taken together these results suggest that CaMKII regulates p21 mRNA expression levels by downregulating the activity of ERK1/2.

3.3. Regulation of p21 transcription

Transcriptional activation of p21 gene is regulated though p53-dependent and -independent mechanisms. We first tested whether the transcriptional activation of the p21 gene was dependent on p53 activity. For this purpose, we analyzed by Western Blot phospho-p53 at Ser 15 together with total p53 and p21 levels in the absence and in the presence of the p53 inhibitor pifithrin-α (PFT-α) (Zhu, et al., 2002b), and analyzed the influence of this inhibitor on cell survival after 72 h of serum withdrawal. Neither total nor phosphorylated (active form (Siliciano, et al., 1997)) p53 levels changed in AD lymphoblasts when compared with control cells despite the increased content of

p21 in AD cells (Fig. 4A). Treatment with PFT-α effectively decreased the levels of phospho-p53 and total p53, but had little effect on p21 levels (Fig. 4A) and didn't overcome the resistance of AD cells to death induced by serum withdrawal (Fig. 4B).

We next focused on p53-independent mechanisms of p21 transcriptional activation. The transcription factor FOXO3a has been shown to mediate the transcriptional transactivation of p21 gene in some type of cells (Hauck, et al., 2007), and it has also been reported that sustained ERK activation results in FOXO3a downregulation (Yang, et al., 2008). Levels of FOXO3a were first determined in control and AD lymphoblasts. As observed in Fig. 5A, AD cells show a higher content of FOXO3a than control cells. FOXO3a is known to be phosphorylated at Ser 294, Ser 344 and Ser 425 by ERK1/2 (Yang, et al., 2008). Phosphorylation of the molecule is required for the export of the transcription factor from the nucleus to be degraded (Obsil and Obsilova, 2008). We could not find a suitable phospho-specific antibody to recognize phosphorylated FOXO3a at these residues, to assess possible differences in phosphorylation status of FOXO3a between control and AD lymphoblasts as the result of their intrinsic differences in ERK1/2 activity (see Fig.1D). Thus, to address this issue, we performed subcellular fractionation experiments to determine whether there is a distinct distribution of FOXO3a in AD cells. We found a preferentially nuclear localization of FOXO3a in the nucleus, which is significantly enhanced in AD lymphoblasts (Fig. 5B). We then aimed to elucidate whether inhibiting ERK1/2 activation by PD98059 or alternatively increasing ERK1/2 phosphorylation with the CaM antagonist CMZ or the CaMKII inhibitor KN-62 impacts the nuclear accumulation of FOXO3a. First of all, Fig. 6A shows the efficacy of these drugs modulating the activity of ERK1/2. Then, subcellular fractionation was performed to assess the nuclear content of FOXO3a under the different experimental conditions. Fig. 6B shows that PD98059 treatment effectively increased the nuclear levels of FOXO3a in control cells, while increasing the activation of ERK1/2 by inhibiting CaMKII by CMZ or KN-62 abrogated the enhanced nuclear levels of AD cells to levels similar to those of control cells (Fig. 6C).

After being exported from the nucleus, phosphorylated FOXO3a is then degraded in the cytosol via an MDM2-mediated ubiquitin-proteasome pathway (Yang, et al., 2008). Moreover, ERK1/2 has been shown to upregulate MDM2 protein expression levels (Phelps, et al., 2005, Ries, et al., 2000). On these grounds, we were interested at elucidating whether control and AD lymphoblasts display distinct MDM2 levels. As shown in Fig. 7, we observed higher levels of MDM2 and ERK1/2 activation in control lymphoblasts when compared with lymphoblasts from AD patients. On the other hand, the inhibition of ERK1/2 activity decreased significantly the cellular content of MDM2 protein (Fig. 7). Thus, it seems that MDM2 may function as the ubiquitin ligase for FOXO3a proteasome degradation in human lymphoblasts. Taken together the results suggest that downregulation of ERK1/2-mediated phosphorylation of FOXO3a in AD lymphoblasts may compromise its transport from the nucleus and its interaction with MDM2, to reduce the degradation of the molecule. The non-phosphorylated FOXO3a accumulated in more extent in the nucleus of AD lymphoblasts, thereby promoting the enhanced transcription of p21.

3.4. Intracellular localization of p21.

The p21 protein has been considered as a paradigm of protein showing completely different nuclear and cytosolic functions (Agell, et al., 2006, Coqueret, 2003). Therefore, we decided to examine whether there are differences in the subcellular localization of p21 in control and AD lymphoblasts. For this purpose, we performed nuclear and cytoplasmic fractionation and analyzed the protein extracts by Western Blot. As shown in Fig. 8, p21 is accumulated in the cytoplasm rather than in nucleus, both in control and AD cells. Nuclear and cytoplasmic content of p21 are higher in AD lymphoblasts in consonance with the increased mRNA p21 levels. Similar results were obtained when cells were immunostained and processed for confocal laser imaging, as shown in Fig. 8.

4. Discusion

In AD lymphoblasts, the constitutive activation of Ca^{2+}/CaM-dependent signaling pathways enhances cell proliferation and survival. Among them, the PI3K/Akt and ERK1/2 seem to play important roles in controlling cell fate depending on growth factor availability (de las Cuevas, et al., 2010). These tumor-like features of lymphoblasts from AD patients were considered as systemic manifestations of the proposed relationship between cellular stress and unscheduled cell cycle entry observed in susceptible neurons in AD (Zhu, et al., 2004). While the CaM-dependent overactivation of PI3K/Akt regulates cell proliferation by enhancing the rate of p27 degradation (Munoz, et al., 2008), here we report that ERK1/2 activation following serum deprivation is negatively

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regulated by CaMKII in AD cells, leading to an increase in FOXO3a activity, thereby enhancing the transcription of p21 and the survival of lymphoblasts from AD patients. Several lines of evidence support this assumption; first, CaMKII and ERK1/2 activation are inversely correlated in immortalized lymphocytes, CaMKII activity is increased in AD cells together with decreased ERK1/2 phosphorylation in the absence of serum; second, CaMKII inhibition restored ERK1/2 phosphorylation and decreased the levels of p21 in AD cells, thus sensitizing AD cells to apoptosis; and third, inhibition of ERK1/2 by PD98059 prevented the serum deprivation-induced death in control cells by increasing the cellular content of FOXO3a and the levels of p21.

FOXO3a is a member of the class O subgroup of the forkhead transcription factor family, which is expressed in many different tissues and involved in mediating the effects of growth factors on several cellular processes including cell cycle progression, glucose metabolism, and apoptosis (Accili and Arden, 2004, Greer and Brunet, 2008, Kops, et al., 2002). The activity of FOXO3a as a transcriptional regulator is primarily, although not exclusively, controlled by its subcellular localization (Vogt, et al., 2005). In the absence of growth- or neurotrophic factors, FOXO3a may localize to the cell nucleus, causing transcriptional induction of a variety of genes (Modur, et al., 2002). Our results indicate that the cellular content of FOXO3a is enhanced in serum-deprived AD lymphoblasts. Under these conditions, FOXO3a accumulated mainly in the nucleus, inducing the transcription of p21 and favoring the cell resistance to serum deprivation. FOXO3a levels appear to be controlled by CaM/CaMKII/ERK1/2-mediated phosphorylation, and subsequent degradation of the molecule in the cytosolic compartment by an MDM2-mediated ubiquitin-proteasome pathway as suggested by the observed decrease in the cellular content of MDM2 protein in AD lymphoblasts.

Experimental evidence supports the idea that important oncogenic kinases such as PI3K/AKT, IKK and ERK1/2 are involved in regulating FOXO3a nuclear localization and activity through phosphorylation at different sites (Hu, et al., 2004, Plas and Thompson, 2003, Yang, et al., 2008). However, in human lymphoblasts, inhibition of PI3K/Akt had no consequences in the transcription of p21 (this manuscript) or in p21 levels and cell survival (Bartolome, et al., 2010). Our data indicate that inhibiting ERK1/2 activity by PD98059 might prevent phosphorylation of FOXO3a, allowing the nuclear accumulation of FOXO3a, increasing its transcriptional activity and blocking the serum withdrawal-induced death in control lymphoblasts.

In addition to the increased transcriptional activation of p21 gene observed in AD lymphoblasts, we also found an increase in the cytosolic content of p21 of AD cells. The cytoplasmic p21 is thought to be a positive modulator of cell survival (Blagosklonny, 2002, Coqueret, 2003).

On the other hand, we did not observe changes in the rate of p21 degradation following serum deprivation. This observation is in contrast with the previously reported increase in p21 degradation in AD lymphoblasts under proliferating conditions (Sala, et al., 2008). Thus, it seems that serum stimulation or withdrawal induced opposite changes in p21 cellular content by affecting protein stability or transcription respectively.

The increased levels of p21 in AD lymphoblasts associated with enhanced resistance to death induced by serum deprivation reported here, are in line with the observation that upregulation of p21 blocked the oxidative stress-induced death of human myeloma U266 cells (Kim, et al., 2001) and rendered resistance to chemotherapy drugs in other types of cancer cells (Gareau, et al., 2011). Thus the increase in p21 cellular content in AD lymphoblasts may confer these cells a survival advantage similar to that described for cancer cells (Weiss, 2003). Increased levels of p21 may protect cells from apoptosis through different mechanisms: p21 can interact and inhibit the activity of caspases (Roninson, 2002), it can also bind to E2F1 and MYC preventing their transcriptional induction of pro-apoptotic proteins (Abbas and Dutta, 2009). On the other hand, p21 can mediate the upregulation of anti-apoptotic proteins (Dotto, 2000). Interestingly, upregulation of p21 has also been associated with blockade of oxidative stress-induced apoptosis in fibroblasts from AD patients (Naderi, et al., 2006). In this sense, it is worth to note that a selective impairment of mechanisms involved in cell death has been previously reported in peripheral cells from AD patients (Eckert, et al., 2001, Morocz, et al., 2002, Naderi, et al., 2006, Uberti, et al., 2002) although there are conflicting results as to whether cells from AD patients are more resistant or more vulnerable to situations that promote cell death. Most likely these discrepancies result from the different cell types and stress-inducing conditions used.

AD cells treated with the CaM antagonist CMZ or the CaMKII inhibitor KN-62 undergo significant apoptosis in the absence of serum in

the culture medium as they do control cells. The effects of these compounds are accompanied by activation of ERK1/2 together with decreased levels of FOXO3a and p21. Inactivation of CaMKII has no effects in control cells suggesting a threshold for CaM activation as the survival signal. Our results are in consonance with previous reports indicating that CaM antagonists specifically resulted in apoptosis in tumorigenic mammary carcinoma cells, but did not affect normal mammary gland-derived epithelial cells (Deb, et al., 2004).

CaM/CaMKII downregulates ERK1/2 activity in human lymphoblasts by a mechanism not yet completely defined. Several mechanisms for CaM/CaMKII modulation of ERK1/2 have been proposed. For example, it was shown in thyroid cancer cells that CaMKII associates and phosphorylates Raf-1, contributing to ERK activation (Illario, et al., 2003). On the other hand, it was reported that CaM negatively regulates the Ras/Raf/MEK/ERK pathway in fibroblasts (Villalonga, et al., 2001). CaMKII can activate in vitro synGAP (Oh, et al., 2004), a Ras inhibitory GTPase expressed in neurons (Kim, et al., 1998), which may inhibit ERK activation. Recently, it has been shown that CaM may prevent the phosphorylation of K-Ras by different kinases and that inactivation of CaM leads to K-Ras activation (Alvarez-Moya, et al., 2011). Apparently, both the C-terminal polylysine region and the farnesylation of K-Ras are important for its specific interaction with CaM (Wu, et al., 2011). Whether there are similar mechanisms operative in human lymphocytes and impaired in AD patients will need further investigation.

In addition to the indirect effect of CaM/CaMKII on cellular content of FOXO3a levels, by preventing its phosphorylation by ERK1/2, and favoring thereafter the transcription of p21, one must consider the possibility that these genes belong to the recently described gene pool, apparently important for adaptive processes, whose expression is highly sensitive to nuclear Ca^{2+}/CaM signaling (Zhang, et al., 2009).

Impaired CaMKII activation has also been detected in AD brain, associated with increased phosphorylation of tau and neurofibrillary tangle formation (McKee, et al., 1990). Similarly, abnormal ERK1/2 activation has been found in degenerating neurons (Zhu, et al., 2002a). ERK1/2 pathway appears to be actively involved in the pathogenesis of AD, by altering hippocampal synaptic plasticity, and contributing to memory deficit (Derkinderen, et al., 1999). On the other hand, increased FOXO3a activity has been found in the brain of the Tg2576 mouse model of AD compared with wild-type mice (Qin, et al., 2008) correlating with increased Aβ1-40 and Aβ1-42 peptide contents. It is also worth mentioning that overexpressed p21 has also been detected in the frontal cortex of AD brains (Engidawork, et al., 2001). Thus, our results obtained in peripheral cells from AD patients demonstrate that dysfunction of CaMKII/ERK1/2/FOXO3a signaling is not restricted to the brain, but rather represents a systemic aspect of the disease. These findings also suggest that changes in B-lymphocytes may contribute to the AD-specific phenotype, in agreement with previous reports highlighting the role of alterations in cells of the immune system in AD pathogenesis (Prinz, et al., 2011). While most of the studies on AD lymphocytes have been focused in detecting disease-specific changes that may serve as biomarkers, it remains to be established the clinical consequences, if any, of the altered response of lymphocytes in AD patients.

In summary, we have provided evidence that in the absence of serum, the survival of AD lymphoblasts depends on the CaMKII-induced downregulation of ERK1/2 activity, leading to increased accumulation of FOXO3a in the nucleus, and therefore to enhanced transcription of p21. The proposed scenario is represented schematically in Fig. 9. Our data indicate that the direct inverse coupling of CaMKII and ERK1/2 and ERK and FOXO3a activity function as key switches in controlling cell fate survival or death depending on growth factors availability.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1

Summary of study population

		Control n=23	AD n=34
Age		75±1	76±5
Age range		(60-83)	(59-89)
Gender	Male	8	15
	Female	15	19

Control, healthy control individuals, no sign of neurological disease; AD, patients with a diagnosis of probable AD.

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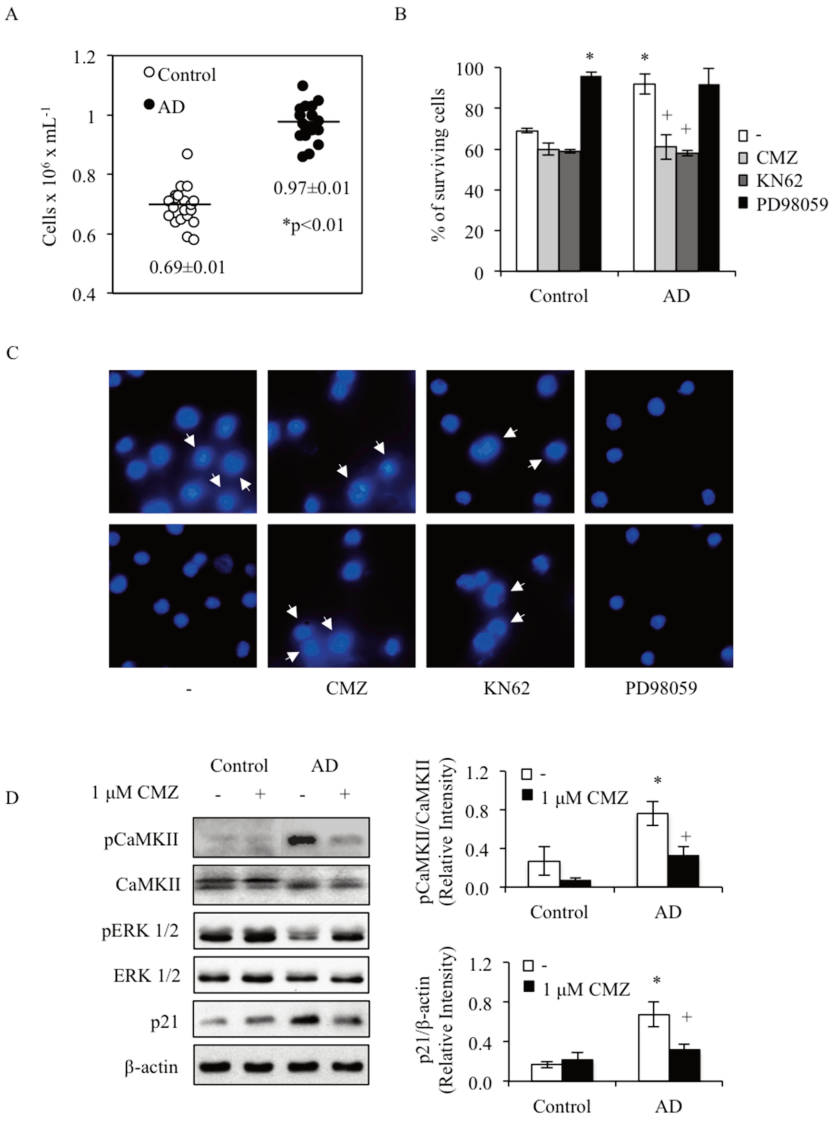
Figures

FIGURE 1



Impaired apoptosis in AD lymphoblasts

Figure 1

Survival of control and AD lymphoblasts in serum-deprived medium. Influence of CaMKII and ERK1/2 on cell survival and p21 content.

A. Scatter plot comparing cell survival following serum deprivation between lymphoblasts derived from control or Alzheimer's disease (AD) patients. Immortalized lymphocytes from control (open symbols) and AD individuals (filled symbols) were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and incubated in serum-free RPMI medium for 72 hours. Cell viability was determined by trypan blue exclusion under inverted phase-contrast microscopy. Statistical significance was determined by the Student's *t*-test. **B**. Percentage of surviving cells, measured as above, after treatment with 1 μM calmidazolium (CMZ), 1 μM KN-62 or 20 μM PD98059. Experimental conditions are identical to those described in A. Data shown are the mean ± SEM of six different experiments carried out with different cell lines, and expressed as percentage of the initial number of cells at day 0. *$p<0.05$ significantly different from untreated control cells. +$p<0.05$ significantly different from untreated AD lymphoblasts. **C**. Representative photomicrograph of DAPI-stained lymphoblasts following 72 hours of serum deprivation in the absence or in the presence of 1 μM CMZ, 1 μM KN-62 or 20 μM PD98059. Arrows show the presence of chromatin condensation/fragmentation indicating apoptosis. No signs of apoptosis were detected in cells treated with PD98059. Treatment of AD cells with CMZ or KN-62, sensitized AD lymphoblasts to death induced by serum deprivation. **D**. Effects of CMZ on CaMKII and ERK1/2 activities and p21 content in control and AD lymphoblasts. Representative immunoblots are shown. Densitometric analyses of phosphorylated CaMKII and p21 content represent the mean ± SEM of four independent observations carried out with different cell lines. *$p<0.05$ significantly different from control cells. +$p<0.05$ significantly different from untreated AD lymphoblasts.

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FIGURE 2

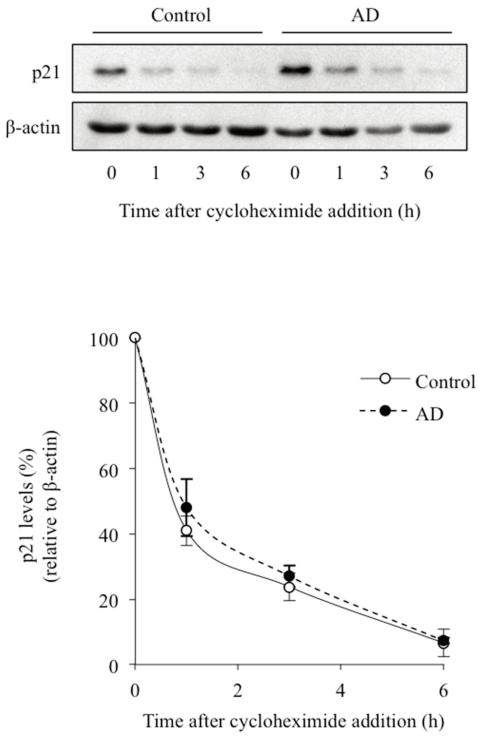

Figure 2

Half-life of p21 in control and AD lymphoblasts.

Lymphoblasts from control and AD subjects were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and incubated in the presence of cycloheximide (20 µg/mL) in serum-free RPMI medium. Cells were harvested 1, 3, and 6 hours thereafter and p21 was detected by immunoblotting. Blots from a representative experiment are shown. The decay of the p21 signal was graphed as a function of time post-cycloheximide addition, using data from different experiments carried out with of cell lines derived from six control and six AD subjects.

Impaired apoptosis in AD lymphoblasts

FIGURE 3

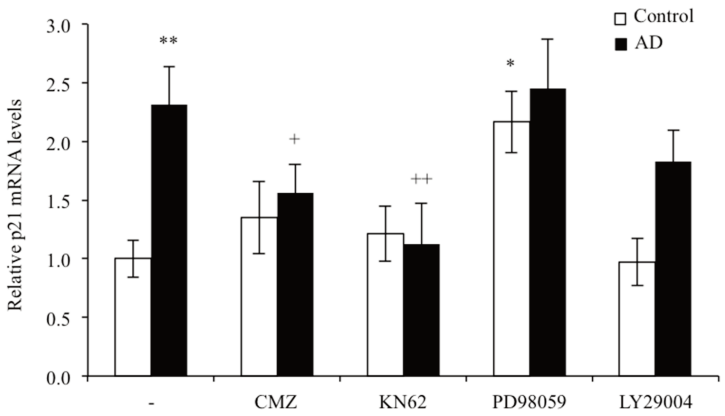

Figure 3

Relative p21 mRNA abundance in control and AD lymphoblasts.

Immortalized lymphoblasts from control and AD individuals were seeded at an initial density of 1 x 10^6 x mL^{-1} and cultured for 24 hours in serum-free RPMI medium as described in legend to Fig. 1 with 1 μM CMZ, 1 μM KN-62, 20μM PD98059 or 1μM Ly294002. Cells were then collected and subjected to quantitative RT-PCR. Relative mRNA levels of the p21 gene in the different experimental conditions were normalized to β-actin expression, and values for control cells were set as one. Values shown are the mean ± SEM for seven different control or AD cell lines. *p<0.05, **p<0.01 significantly different from control cells; +p<0.05, ++p<0.01 significantly different from untreated AD cells

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FIGURE 4

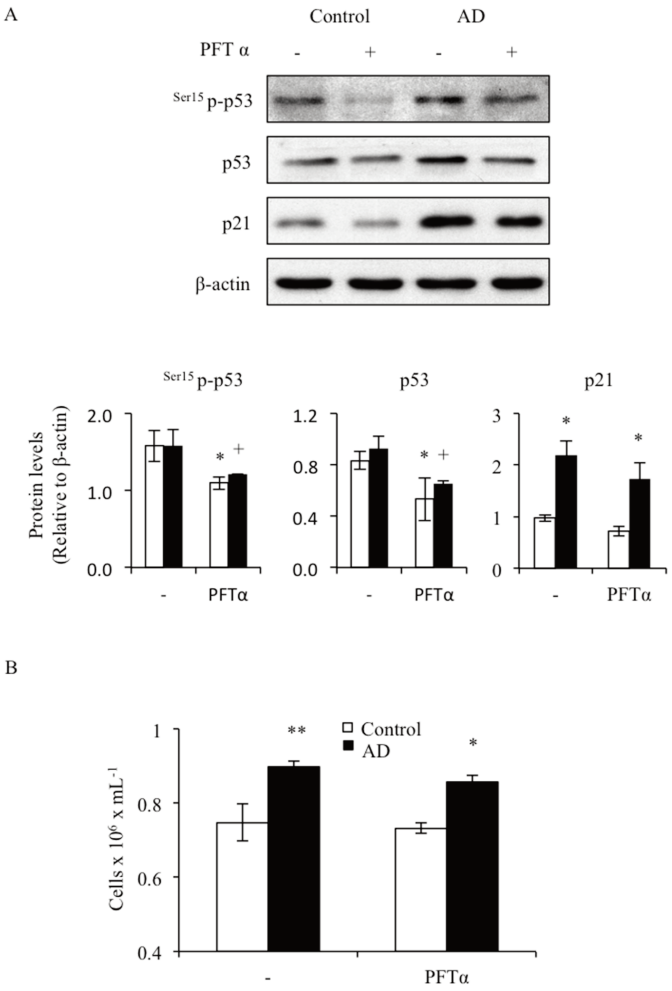

Figure 4

Effects of pifithrin-α (PFT-α) on p53 and p21 content and in survival of control and AD lymphoblasts.

Lymphoblasts from control and AD subjects were incubated in serum-free RPMI medium for 72 hours in the absence or in the presence of 30 μM PFT-α. **A.** To assess the effects of the drug on p21 and p53 content and in p53 phosphorylation in Ser 15, cell extracts for Western Blot were prepared at 24 hours. A representative immunoblot is shown. Densitometric analyses of p21, p-p53 (Ser 15) and total p53 content represent the mean ± SEM from different observations carried out with four different cell lines. *p<0.05 significantly different from untreated control cells, +p<0.05 significantly different from untreated AD cells. **B.** Cell viability was determined by trypan blue exclusion under inverted phase-contrast microscopy. Data shown represent the mean ± SEM from different observations carried out with five different cell lines.*p<0.05, **p<0.01 significantly different from untreated control cells.

Impaired apoptosis in AD lymphoblasts

FIGURE 5

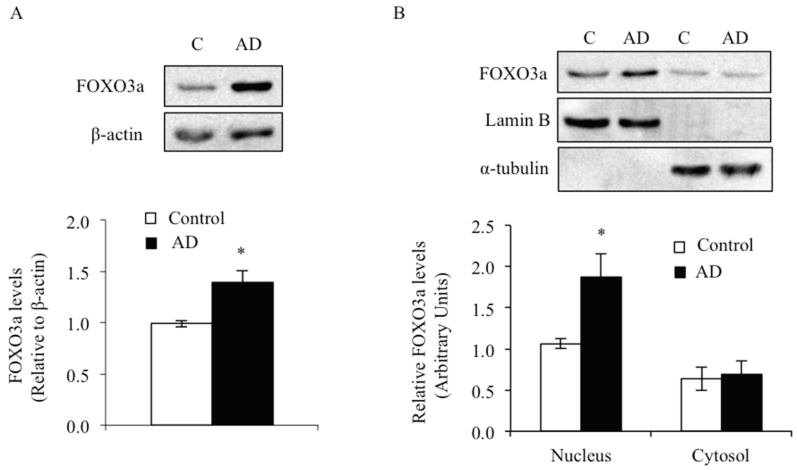

Figure 5

Relative FOXO3a abundance and subcellular localization in control and AD cells

A. Lymphoblasts from control and AD cells were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and serum-starved for 24 hours. At that time, cell extracts for Western Blotting were prepared. A representative immunoblot is shown. Densitometric analyses of FOXO3a content represent the mean ± SEM of different experiments performed with ten different control or AD subjets. *p<0.05 significantly different from control cells. **B.** Experiments were performed under the same conditions described in A. In this case, cells were fractionated into cytosolic and nuclear fractions and subjected to Western Blot. Lamin B and α-tubulin were used as control of purity and loading of nuclear and cytoplasmic protein extracts, respectively. Data represent the mean ± SEM of different experiments carried out with eight different cell lines derived from control or AD patients. *p<0.05, significantly different from control nucleus.

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FIGURE 6

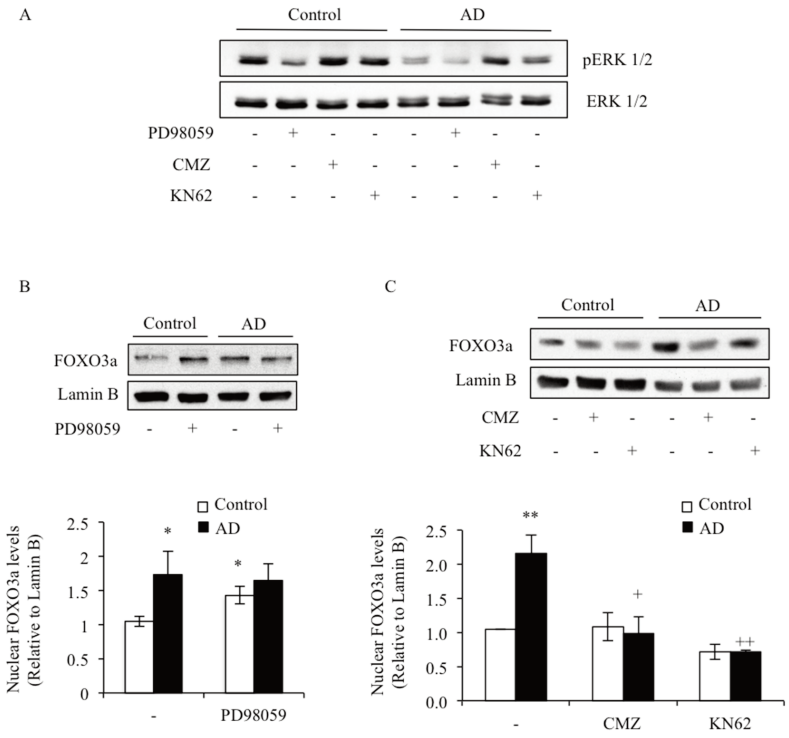

Figure 6

Influence of CaMKII and ERK1/2 on FOXO3a nuclear localization

A. Cells were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and incubated in serum-free RPMI medium in the absence or in the presence of 20 µM PD98059, 1 µM CMZ or 1 µM KN-62 for 24 hours. Part of these cells was used to prepare whole-cell extracts, which were subjected to Western blot to check the effects of these drugs on ERK phosphorylation, as shown in a representative immunoblot. **B.** The rest of the cells were fractionated to get the nuclear fraction. Nuclear FOXO3a abundance as determined by Western Blot in the absence or in the presence of PD98059 was calculated with data derived from experiments using eight different cell lines. *p<0.05, significantly different from untreated control. **C**. Under the same conditions as B, nuclear FOXO3a abundance as determined by Western Blot was calculated in the absence or in the presence of CMZ and KN62. Representative immunoblots are shown. Four different cell lines derived from control or AD subjects were used to perform the experiments. *p<0.01, significantly different from untreated control; +p<0.05, significantly different from untreated AD; +p<0.01, significantly different from untreated AD. Densitometric analyses represent in all cases the mean ± SEM.

Impaired apoptosis in AD lymphoblasts

FIGURE 7

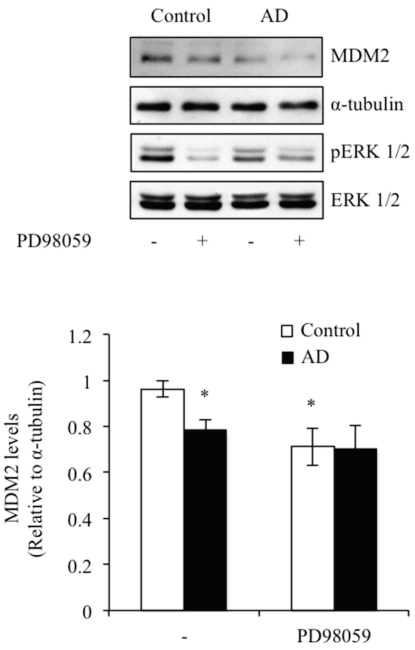

Figure 7

Effects of ERK1/2 on MDM2 content in control and AD lymphoblasts.

Cells were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and incubated in serum-free RPMI medium in the absence or in the presence of 20 μM PD98059 for 24 hours, when they were collected to prepare whole-cell extracts for Western Blot. A representative immunoblot is presented. Densitometric analyses of MDM2 content represent the mean ± SEM of different experiments carried out with six different cell lines from control or AD patients. *p<0.05 significantly different from untreated control.

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FIGURE 8

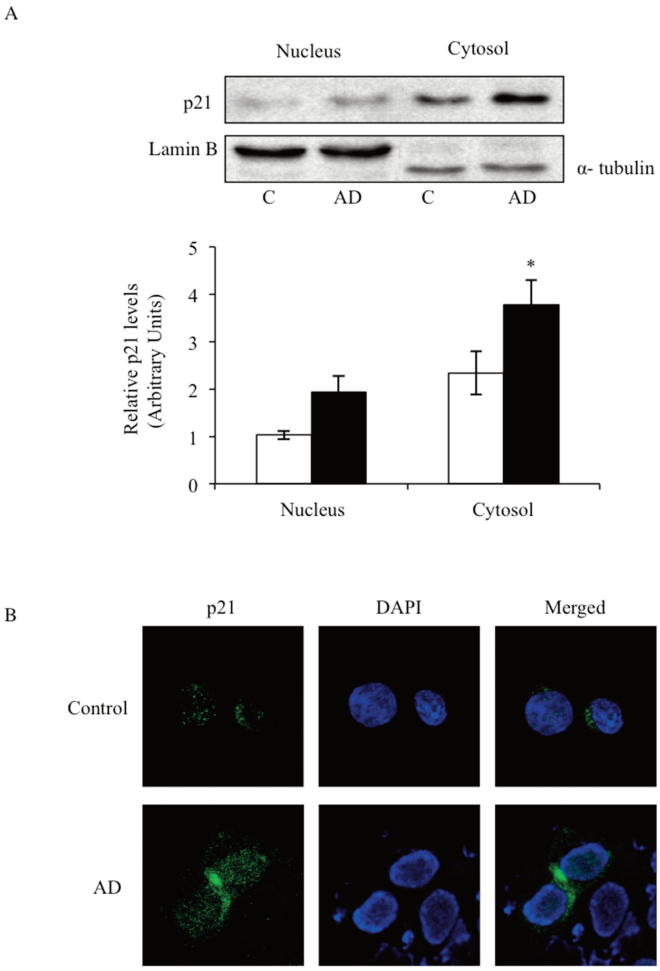

Figure 8

Subcellular localization of p21 in control and AD lymphoblasts.

A. Lymphoblasts from control and AD patients were seeded at an initial density of 1 x 10^6 cells x mL^{-1}, incubated in the absence of serum for 24 hours, and then fractionated to determine by immunoblot the subcellular localization of p21. Antibodies to α-tubulin and to lamin B were used as control of purity and loading of cytoplasmic and nuclear protein extracts, respectively. A representative immunoblot is shown, whereas the densitometric analysis is presented below. Data represent mean ± SEM of eight experiments. *p<0.01 significantly different from cytosol of control cells. **B.** p21 localization was also studied by confocal microscopy. Under the same conditions than A, cells were stained with anti-p21 antibody followed by secondary antibody labeled with Alexa Fluor 488. DAPI was used for nuclear staining. Merged images depict a predominant cytosolic localization for p21, more evident in AD cells. Magnification: 63x.

Impaired apoptosis in AD lymphoblasts

FIGURE 9

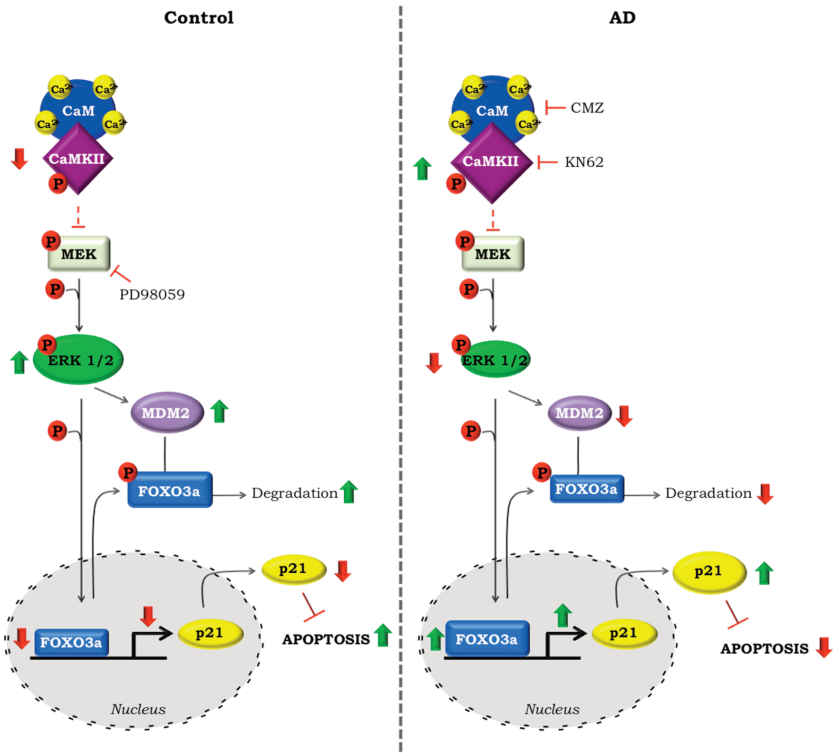

Figure 9
Diagram summarizing the role of CaMKII/ERK1/2/FOXO3a pathway in regulating p21 content and survival of AD lymphoblasts.

In control cells, serum withdrawal promotes ERK1/2 activation, which then presumably induces phosphorylation of FOXO3a and its posterior degradation in the cytosolic compartment in an MDM2-mediated manner. In AD cells, ERK1/2 activation is downregulated by the enhanced CaMKII activity. Reduced ERK1/2 activity partially prevents phosphorylation and degradation of FOXO3a increasing thereby the transcriptional activation of p21, which in turn protects cells from apoptosis.

Alteraciones en la expresión de genes relacionados con el ciclo celular en cerebro y linfocitos de un modelo de ratón transgénico de EA (APP/PS1)

Artículo #4 : **Noemí Esteras**, Fernando Bartolomé, Carolina Alquézar, Desiré Antequera, Úrsula Muñoz, Eva Carro, Ángeles Martín-Requero (2012). "Altered cell cycle-related gene expression in brain and lymphocytes from a transgenic mouse model of Alzheimer's disease (APP/PS1)" **Eur J Neurosci**. doi:10.1111/j.1460.9568.2012.08178.x

Resumen

La alteración del control del ciclo celular en neuronas maduras es un evento importante en la neuropatogénesis de la EA. El intento de reactivación del ciclo celular de las neuronas post-mitóticas, acompañado de la expresión aberrante de marcadores del ciclo, se ha observado en el cerebro de pacientes y también en muchos modelos animales de la enfermedad, a pesar de que la mayoría de éstos no desarrollan todas las características de la EA, como la muerte neuronal. También se ha observado en células periféricas de pacientes, como fibroblastos y linfocitos. En este trabajo, hemos estudiado simultáneamente posibles alteraciones del ciclo celular en cerebro y linfocitos del modelo murino de EA APP/PS1 a los doce meses de edad. Para ello, hemos empleado la tecnología RT²Profiler™ PCR Array, basada en arrays de PCR, para analizar la expresión de 84 genes diferentes relacionados con el ciclo celular. El estudio se ha realizado en linfocitos y cerebro y nos ha permitido identificar un buen número de genes en los dos tejidos de los ratones transgénicos cuya expresión estaba alterada respecto a los ratones control. Además, hemos observado que los linfocitos de los ratones transgénicos muestran una mayor incorporación de 5'-bromo-2'-deoxiuridina (BrdU) en el ADN que los linfocitos de los ratones control cuando son estimulados con mitógenos, indicando una mayor respuesta proliferativa. Por último, las neuronas corticales de los ratones APP/PS1 exhiben una mayor expresión del marcador de proliferación PCNA y del inhibidor de Cdk Cdkn2a o p16, como muestra la inmunohistoquímica. Todos estos datos avalan las alteraciones en el control del ciclo celular como un mecanismo importante en la EA, además de validar el uso de células periféricas como modelo de estudio de las bases moleculares de la enfermedad.

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Altered cell cycle-related gene expression in brain and lymphocytes from a transgenic mouse model of Alzheimer's disease [amyloid precursor protein/presenilin 1 (PS1)]

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Keywords: Alzheimer's disease, amyloid precursor protein/*PS1 mice, cell cycle, gene expression, lymphocytes, polymerase chain reaction arrays*

Abstract

Cumulative evidence indicates that aberrant re-expression of many cell cycle-related proteins and inappropriate neuronal cell cycle control are critical events in Alzheimer's disease (AD) pathogenesis. Evidence of cell cycle activation in post-mitotic neurons has also been observed in murine models of AD, despite the fact that most of these mice do not show massive loss of neuronal bodies. Dysfunction of the cell cycle appears to affect cells other than neurons, as peripheral cells, such as lymphocytes and fibroblasts from patients with AD, show an altered response to mitogenic stimulation. We sought to determine whether cell cycle disturbances are present simultaneously in both brain and peripheral cells from the amyloid precursor protein (APP)/presenilin 1 (PS1) mouse model of AD, in order to validate the use of peripheral cells from patients not only to study cell cycle abnormalities as a pathogenic feature of AD, but also as a means to test novel therapeutic approaches. By using cell cycle pathway-specific RT^{2}Profiler™ PCR Arrays, we detected changes in a number of cell cycle-related genes in brain as well as in lymphocytes from APP/PS1 mice. Moreover, we found enhanced 5′-bromo-2′-deoxyuridine incorporation into DNA in lymphocytes from APP/PS1 mice, and increased expression of the cell proliferation marker proliferating cell nuclear antigen (PCNA), and the cyclin-dependent kinase (CDK) inhibitor Cdkn2a, as detected by immunohistochemistry in cortical neurons of the APP/PS1 mice. Taken together, the cell cycle-related changes in brain and blood cells reported here support the mitosis failure hypothesis in AD and validate the use of peripheral cells as surrogate tissue to study the molecular basis of AD pathogenesis.

Introduction

Mounting evidence indicates that aberrant re-expression of many cell cycle-related proteins and inappropriate cell cycle control in specific vulnerable neurons in Alzheimer's disease (AD) are important components in AD pathogenesis (Arendt, 2003). Neuronal changes supporting alterations to cell cycle control in the etiology of AD include the ectopic expression of cell cycle markers, cytoskeletal alterations (Copani *et al.*, 2001; Nagy, 2005) and full or partial DNA replication (Yang *et al.*, 2001). A more than diploid DNA content has been detected in differentiated neurons from subjects with AD (Mosch *et al.*, 2007; Arendt *et al.*, 2009), associated with decreased viability (Zekanowski & Wojda, 2009; Arendt *et al.*, 2010). These events occur early in the progression of AD (Yang *et al.*, 2003; Yang & Herrup, 2007), suggesting that cell cycle-induced death is a central mechanistic feature of the disease (Herrup & Yang, 2007).

Work from our laboratory and others has shown cell cycle disturbances in peripheral cells from patients with AD, suggesting that dysfunction of the cell cycle is a more general phenomenon affecting cells other than neurons (Stieler *et al.*, 2001; Urcelay *et al.*, 2001; Nagy *et al.*, 2002). Indeed, a recent multivariate analysis of cell cycle activity in AD lymphocytes has pointed out that the disturbed mitogenic response of peripheral cells from patients with AD may aid the current differential diagnosis of AD (Stieler *et al.*, 2012).

Cell cycle-related events have also been reported in murine models of AD, although these mice do not show significant loss of neuronal

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5 Alteraciones en la expresión de genes relacionados con el ciclo celular en cerebro y linfocitos de un modelo de ratón transgénico de EA (APP/PS1)

TABLE 1. Sequences of oligodeoxyribonucleotide primers used for quantitative real-time PCR

	Primer sequence	
Gene	Forward (5′ → 3′)	Reverse (5′ → 3′)
Abl1	AATCCAAGAAGGGGCTCTCT	GCCTCTGCAGGGCTTCTT
Actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
Ccnf	GGCTTTCTGTTGGGGACAT	CCAGGTACTTGAGGTGGGAGT
Cdk4	TCAGTGGTGCCAGAGATGG	GGAAGGCAGAGATTCGCTTA
Cdk5rap1	GAAGCAGCCGTGTACTGGAT	ATGCCACATATGCTTCTCTTGA
Cdkn1a	TCCACAGCGATATCCAGACA	GGACATCACCAGGATTGGAC
Cdkn2a	CGACGGGCATAGCTTCAG	GCTCTGCTCTTGGGATTGG
Chek1	ACTGGGATTTGGTGCAAACT	CAGTTATTCTATTCACAGCAAGTTGAA
E2f1	TGCCAAGAAGTCCAAGAATCA	CTTCAAGCCGCTTACCAATC
Gpr132	AGGAGCCACCATGAGATCAG	TGCTCTGAAGAACGGAGGTAA
Mcm4	ATGCCCCGACTGAGTGAG	CGGCTACTCCCAATCTTCCT
Myb	TGTCAACAGAGAACGAGCTGA	GCTGCAAGTGTGGTTCTGTG
Nek2	ACTGCAGGATCGAGAGCAAG	CGAATACAAAGTTCACGTTCCTT
Nfact1	TCCAAAGTCATTTTCGTGGA	CTTTGCTTCCATCTCCCAGA
Sesn2	ACATCCACTGCGTCTTTGG	CGTCTTGATATAGATTTTGAGGTTCC

Probes were designed using the Universal Probe Library for Human (Roche Applied Science).

bodies (Yang *et al.*, 2006; Malik *et al.*, 2008). It appears that cell cycle-positive neurons are committed to die, but they can survive in a compromised manner until they are hit by another detrimental factor (Zhu *et al.*, 2004b, 2007). However, it has been reported that the intracerebral injection of adeno-associated viral particles containing mutations of tau or amyloid precursor protein (APP) leads to the ectopic expression of cell cycle-related genes (Jaworski *et al.*, 2009), reinforcing the hypothesis of post-mitotic neurons forced to enter the cell cycle in AD.

On these grounds, we considered it interesting to evaluate if cell cycle disturbances are present in both brain and peripheral cells from a transgenic mouse model of AD. To this end, we performed a comparative analysis of the expression of cell cycle-related genes in brain and lymphocytes from APP/presenilin 1 (PS1) mice. This murine model of AD typically expresses sufficiently high levels of human APP (hAPP) and amyloid-β to ensure amyloid deposition (Antequera *et al.*, 2009) and apoptotic cell death in the cerebral cortex of APP/PS1 mice (Spuch *et al.*, 2010).

Data from our study indicate that the APP/PS1 mice show differential expression of cell cycle-related genes in both brain and peripheral blood mononuclear cells (PBMCs). In accordance with these observations, we found enhanced 5′-bromo-2′-deoxyuridine (BrdU) incorporation in mouse lymphocytes as well as the expression of cell cycle-related proteins in cortical neurons. Taken together, these findings support the role of cell cycle failure in AD pathogenesis, as well as the fact that cell cycle dysfunction also occurs at the systemic level.

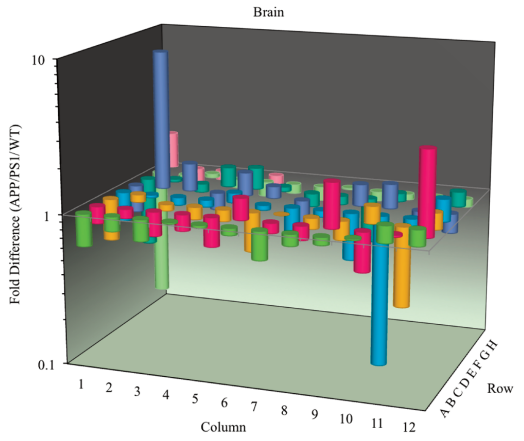

FIG. 1. Cell cycle-specific PCR Array analysis in brain from wild-type (WT) and APP/PS1 mice. Three-dimensional chart taken from SABiosciences analysis software (see Materials and methods) showing the fold difference in expression levels for 84 cell cycle-related genes comparing WT brain (control sample) and APP/PS1 brain (test sample). Individual genes with a greater than 1.5-fold expression change can be found in Table 2.

Materials and methods

Mutant mice

All procedures with animals were specifically approved by the Bioethical and Biosafety Commission of the Center for Biological Research (CIB, CSIC), and carried out in accordance with the protocols issued, which followed national (normative 1201/2005) and international recommendations (directive 2010/63/EU from the European Communities Council). Double-transgenic APP/PS1 mice (male, 12 months of age), a cross of the Tg2576 (overexpressing human APP 695 with double mutations at KM670/671NL) and mutant PS1 (M146L) mice, based on the C57BJ6 phenotype, were used as a model of AD amyloidosis (Holcomb *et al.*, 1998). Non-transgenic C57BJ6 littermates were used as controls.

Tissue harvesting

Mice were anesthetized with a lethal dose of Fatal-Plus (0.22 mL/kg) (Vortech Pharmaceutical, Dearborn, MI, USA) and perfused transcardially with ice-cold saline buffer. The brain was removed and the hippocampus and cerebral cortex were dissected from the right hemibrain and snap-frozen in isopentane containing dry ice and stored at −80 °C. The left hemibrain was fixed in phosphate-buffered 4% paraformaldehyde, pH 7.4, for 5 h at 4 °C. After fixation, the hemibrains were cryoprotected in 20% sucrose solution for 16 h at 4 °C and embedded in TissueTek, frozen in isopentane containing dry ice, and stored at −80 °C.

Alteraciones en la expresión de genes relacionados con el ciclo celular en cerebro y linfocitos de un modelo de ratón transgénico de EA (APP/PS1)

TABLE 2. Mouse PCR Array data showing < -1.5-fold and > 1.5-fold cell cycle RNA expression changes in brain of APP/PS1 mice compared with control mice

Gene name	Symbol	GenBank	Fold change	Cell cycle function
Meiotic recombination 11 homolog A (*Saccharomyces cerevisiae*)	Mre11a	NM_018736	−593	S phase and DNA replication
NIMA (never in mitosis gene a)-related expressed kinase 2	Nek2	NM_010892	8.46	M phase
Schlafen 1	Slfn1	NM_011407	−7.26	Checkpoint and arrest
Cyclin-dependent kinase inhibitor 2A	Cdkn2a	NM_009877	3.55	Checkpoint and arrest
G protein-coupled receptor 132	Gpr132	NM_019925	−3.34	G1 phase and G1/S transition
Antigen identified by monoclonal antibody Ki 67	Mki67	XM_133912	−2.03	S phase and DNA replication
CDK5 regulatory subunit-associated protein 1	Cdk5rap1	NM_025876	1.98	Checkpoint and arrest
Sestrin 2	Sesn2	NM_144907	−1.97	Checkpoint and arrest
Checkpoint kinase 1 homolog (*Saccharomyces pombe*)	Chek1	NM_007691	−1.88	Checkpoint and arrest
Cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	NM_007669	−1.83	Checkpoint, arrest and regulation
E2F transcription factor 1	E2f1	NM_007891	−1.81	Regulation
Glucuronidase, beta	Gusb		1.74	Glucuronidase
Minichromosome maintenance-deficient 4 homolog (*S. cerevisae*)	Mcm4	NM_008565	−1.65	S phase and DNA replication
v-abl Abelson murine leukemia oncogene 1	Abl1	NM_009594	−1.64	Regulation
Cyclin F	Ccnf	NM_007634	−1.56	Regulation
Inhibin alpha	Inha	NM_010564	−1.56	Checkpoint, arrest and negative regulation
Calcium/calmodulin-dependent protein kinase II, alpha	Camk2a	NM_177407	−1.54	G1 phase and G1/S transition
Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	Nfatc1	NM_016791	1.53	G1 phase and G1/S transition
Myeloblastosis oncogene	Myb	NM_033597	−1.52	G1 phase and G1/S transition
Pescadillo homolog 1, containing BRCT domain (zebrafish)	Pes1	NM_022889	−1.51	M phase

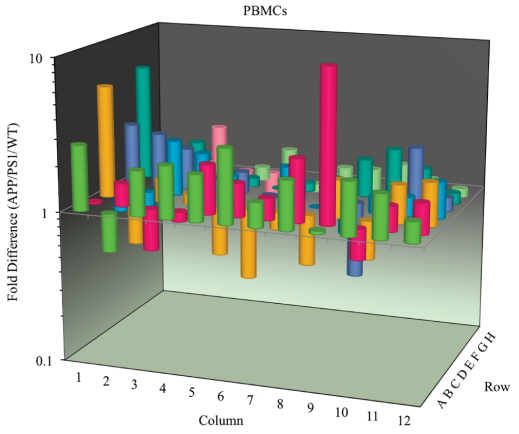

FIG. 2. Cell cycle-specific PCR Array analysis in PBMCs from wild-type (WT) and APP/PS1 mice. PBMCs obtained from pooled blood from four WT or APP/PS1 mice were used to prepare the mRNA for the PCR Array analysis. Three-dimensional chart taken from SABiosciences analysis software (see Materials and methods) showing the fold difference in expression levels for 84 cell cycle-related genes comparing WT lymphocytes (control sample) and APP/PS1 lymphocytes (test sample). Individual genes with a greater than 1.5-fold expression change can be found in Table 3.

Peripheral blood mononuclear cell isolation

Peripheral venous blood (1 mL) from each mouse was obtained by puncture with heparinized syringes and pooled from four animals. PBMCs were isolated by Lymphoprep™ density gradient centrifugation according to the manufacturer's instructions (Axis-Shield Po CAS, Oslo, Norway). Cells were washed twice with phosphate-buffered saline, counted and resuspended at the desired concentration.

Determination of lymphocyte proliferation

The PBMCs were incubated in Roswell Park Memorial Institute (RPMI) medium containing mitogens for 3 days. Cell proliferation was assessed by the BrdU incorporation method using an enzyme-linked immunoassay kit (Roche, Madrid, Spain). Cells (5000 cells/-well) were seeded in 96-well microtiter plates. BrdU (10 μM) was added at 4 h before the end of the interval of measurement. The cells were fixed with pre-cooled 70% ethanol during 30 min at −20 °C and incubated with nucleases following the manufacturer's recommendations. Cells were then treated for 30 min at 37 °C with peroxidase-conjugated anti-BrdU antibody. The excess of antibody was removed by washing the cells three times followed by the addition of substrate solution. Absorbance was measured at 405 nm with a reference wavelength of 492 nm using a microplate reader.

RNA extraction

RNA was extracted from pooled PBMCs from four mice using the RNeasy Mini kit (Qiagen, Hilden, Germany) including column DNase I digestion according to the manufacturer's recommendations. To isolate RNA from individual brains (30 mg of pooled cortex and hippocampus from each mouse was used), the same protocol was performed, adding a previous step with Trizol Reagent (Invitrogen). RNA yields were quantified spectrophotometrically and the RNA quality was checked by the A260 : A280 ratio and with the Bioanalyzer 2100 (Agilent Technologies Inc., Palo Alto, CA, USA).

RT²Profiler™ PCR array

Mouse cell cycle (PAMM-020A) focused pathway arrays (SABiosciences, Frederick, MD, USA) in 96-well plate format were used to assay gene expression changes. Samples were prepared from pooled RNA extracted from the hippocampus and cortex, as well as PBMCs from the pooled blood of four different control or

5 Alteraciones en la expresión de genes relacionados con el ciclo celular en cerebro y linfocitos de un modelo de ratón transgénico de EA (APP/PS1)

TABLE 3. Mouse PCR Array data showing < -1.5-fold and >1.5-fold cell cycle RNA expression changes in PBMCs of APP/PS1 mice compared with control mice

Gene name	Symbol	GenBank	Fold change	Cell cycle function
CDK5 regulatory subunit-associated protein 1	Cdk5rap1	NM_025876	10.69	Checkpoint and arrest
Protamine 1	Prm1	NM_013637	5.79	M phase
Checkpoint kinase 1 homolog (*S. pombe*)	Chek1	NM_007691	5.46	Checkpoint and arrest
Peripheral myelin protein	Pmp22	NM_008885	−3.10	Checkpoint and arrest
Breast cancer 2	Brca2	NM_009765	3.09	M phase, checkpoint, arrest and regulation
E2F transcription factor 1	E2f1	NM_007891	−2.83	Regulation
v-abl Abelson murine leukemia oncogene 1	Abl1	NM_009594	2.69	Regulation
Cyclin-dependent kinase 4	Cdk4	NM_009870	2.58	Regulation
Myeloblastosis oncogene	Myb	NM_033597	2.53	G1 phase and G1/S transition
Protein phosphatase 2 (formerly 2a) regulatory subunit B″, alpha	Ppp2r3a	NM_918708	2.50	G1 phase and G1/S transition
Microtubule-actin cross-linking factor 1	Macf1	XM_884922	2.31	Checkpoint and arrest
NIMA (never in mitosis gene a)-related expressed kinase 2	Nek2	NM_010892	2.27	M phase
Ataxia telangiectasia mutated homolog (human)	Atm	NM_007499	2.23	Negative regulation
Cyclin A1	Ccna1	NM_007628	2.23	M phase and regulation
Stratifin	Sfn	NM_018754	2.14	Checkpoint, arrest and regulation
Cyclin F	Ccnf	NM_007634	2.12	Regulation
E2F transcription factor 3	E2f3	NM_010093	−2.11	Regulation
Dystonin	Dst	NM_134448	−2.10	Checkpoint and arrest
Breast cancer 1	Brca1	NM_009764	2.03	Negative regulation
CDC28 protein kinase 1b	Cks1b	NM_016904	−2.00	Checkpoint, arrest and regulation
Amyloid-β (A4) precursor-binding protein, family B, member 1	Apbb1	NM_009685	1.99	Checkpoint and arrest
MAD2 (mitotic arrest-deficient, homolog)-like 1 (yeast)	Mad2l1	NM_019499	1.98	Checkpoint and arrest
Heat shock protein 90 kDa alpha (cytosolic), class B member 1	Hsp90ab1		1.98	Heat shock protein
Cyclin A2	Ccna2	NM_009828	1.95	Regulation
Hus 1 homolog (*S. pombe*)	Hus1	NM_008316	1.92	Checkpoint and arrest
Cyclin D1	Ccnd1	NM_007631	−1.88	Regulation
RAD21 homolog (*S. pombe*)	Rad21	NM_009009	1.87	M phase
Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent-1	Nfatc1	NM_016791	1.86	G1 phase and G1/S transition
Adenylate kinase 1	Ak1	NM_021515	−1.80	Checkpoint and arrest
Minichromosome maintenance-deficient 4 homolog (*S. cerevisae*)	Mcm4	NM_008565	1.80	S phase and DNA replication
Growth arrest and DNA-damage-inducible 45 alpha	Gadd45a	NM_007836	−1.79	Checkpoint, arrest and regulation
G protein-coupled receptor 132	Gpr132	NM_019925	1.78	G1 phase and G1/S transition
E2F transcription factor 4	E2f4	NM_148952	1.77	Regulation
Sestrin 2	Sesn2	NM_144907	1.76	Checkpoint and arrest
RAD51 homolog (*S. pombe*)	Rad51	NM_011234	1.74	S phase and DNA replication
Mdm2, transformed 3T3 cell double minute p53 binding protein	Mtbp	NM_134092	1.73	G1 phase and G1/S transition
Cell division cycle 25 homolog A (*S. pombe*)	Cdc25a	NM_007658	1.67	M phase
Telomeric repeat-binding factor 1	Terf1	NM_009352	1.67	M phase
Src homology 2 domain-containing transforming protein C1	Shc1	NM_011368	1.65	M phase
Cyclin-dependent kinase inhibitor 2A	Cdkn2a	NM_009877	1.59	Checkpoint and arrest
Cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	NM_007669	−1.58	Checkpoint, arrest and regulation
Integrin beta 1 (fibronectin receptor beta)	Itgb1	NM_010578	−1.58	G1 phase, G1/S transition and regulation
Transformation-related protein 63	Trp63	NM_011641	1.53	Negative regulation

By using cell cycle pathway-specific RT^{2}Profiler™ PCR Arrays, we detected changes in a number of cell cycle-related genes in brain and lymphocytes from APP/PS1 mice. Moreover, we found enhanced BrdU incorporation into DNA in lymphocytes from APP/PS1 mice, and increased expression of cell cycle-related proteins, as detected by immunohistochemistry in cortical neurons of the APP/PS1 mice. These observations support the mitosis failure hypothesis in AD and demonstrate the existence of systemic manifestations of the disease.

APP/PS1 mice. Samples were added to the reaction plates following the manufacturer's instructions and the experiment was performed on a Bio-Rad iQ5 system. Analysis was carried out using the spreadsheet provided by SABiosciences. The absence of DNA contamination and efficiency of amplification were confirmed by the analysis software provided. The gene functions listed in Tables 2 and 3 were obtained from the RT^{2}Profiler™ PCR Array product specification sheets.

Quantitative real-time reverse transcription-polymerase chain reaction

A two-step real-time reverse transcription-polymerase chain reaction (PCR) was used to measure the expression of candidate genes. Isolated total RNA (1 μg) was used to synthesize cDNA in a 20 μL reaction with the Superscript III Reverse Transcriptase kit (Invitrogen). Quantitative real-time PCR was performed in triplicate using TaqMan Universal PCR MasterMix No Amperase UNG (Applied Biosystems) reagent according to the manufacturer's protocol. Primers were designed with the Universal ProbeLibrary (Roche) and used at a final concentration of 20 μM. The sequences of the forward and reverse primers used are listed in Table 1.

Quantitative real-time PCR was performed in the Bio-Rad iQ5 system using a thermal profile of an initial 5 min melting step at 95 °C followed by 40 cycles at 95 °C for 10 s and 60 °C for 60 s.

The relative mRNA levels of the genes of interest were normalized to β-actin expression using the simplified comparative threshold cycle delta, cycle threshold (CT) method [$2^{-(\Delta CT \text{ gene of interest } - \Delta CT \text{ actin})}$].

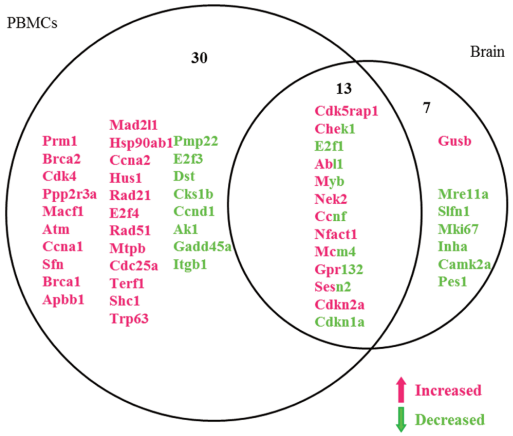

FIG. 3. Comparison between gene expression in PBMCs and brain from wild-type and APP/PS1 mice. Genes that were notably upregulated (red) or downregulated (green) were clustered into a Venn diagram. There were only 13 altered common genes between these two tissues. The names of these genes can be found in Tables 2 and 3.

Fluorescence immunolabeling

Fixed brains were cut on a vibratome (Leica Microsystems) at 50 μm, and tissue sections were collected in cold phosphate buffer, 0.1 M, and incubated overnight with primary antibodies at 4 °C. All primary antibodies were diluted in phosphate buffer, 0.1 M, containing 0.5% bovine serum albumin and 0.5% Triton X-100. The primary antibodies used were: mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (1 : 200, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-p16 sc-1207 (1 : 100, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-calbindin (1 : 500, Swant), mouse monoclonal anti-βIII tubulin (1 : 1000, Promega) and mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (1:500, Sigma). The secondary antibodies used were: donkey anti-mouse IgG 488 (1 : 1000, FluoProbes®, Interchim), and Texas Red goat anti-rabbit IgG antibody (1 : 1000, Jackson Immunoresearch, West Grove, USA). Images were captured using a Zeiss LSM 510 Meta scanning laser confocal microscope (Carl Zeiss Microimaging, GmbH). The specificity of the staining was tested by omitting primary antibodies from the incubation solution. To assess the number of PCNA-positive or p16-positive labeled neurons, we took every sixth brain section containing the frontal cortex (between 2.5 and 3.4 mm anterior to the bregma).

Statistics

Statistical analysis was performed with GRAPHPAD PRISM v. 5 for Macintosh (GraphPad Software, La Jolla, CA, USA). All the data represent the mean ± SEM. All statistical analyses comprised simple comparisons of the means of the same variable between two independent groups, wild-type and APP/PS1. Therefore, an unpaired two-tailed Student's *t*-test was chosen for the determination of significance, and results are reported with the *t*-statistic value, with degrees of freedom as subscript, followed by the *P*-value. The significance level was set at 0.05. Quantitative reverse transcription-PCR analyses in lymphocytes were performed with pooled samples and therefore no statistical analysis was performed.

Results

Altered expression of cell cycle-related genes in brain and peripheral blood mononuclear cells in wild-type and amyloid precursor protein/presenilin 1 mice

We used cell cycle pathway-specific RT^{2}Profiler™ PCR Arrays (see Materials and methods) to quantify and compare the expression of cell cycle-related genes with high sensitivity. We used RNA extracted from the pooled cortex and hippocampus from four control and four APP/PS1 mice. We compared the expression levels of 84 genes that regulate the cell cycle, including transitions between each of the phases, DNA replication, checkpoints and arrest. Twenty of the 84 genes had expression levels that were increased or decreased by more than 1.5-fold in APP/PS1 brain (Fig. 1; Table 2). The array identified changes in genes associated with many different stages of the cell cycle rather than one specific stage (Table 2). The same study was carried out in RNA extracted from pooled PBMCs from these mice, finding that 43 out of 84 genes showed changes larger than 1.5-fold (Fig. 2; Table 3). The comparison of gene expression between brain and PBMCs shows that only 13 genes were notably altered in both tissues (Fig. 3). Cdk5rap1, E2f1, Nek2, Nfact1, Cdkn2a and Cdkn1a were upregulated or downregulated (E2f1, Cdkn1a) in both tissues, whereas Chek1, Abl1, Myb, Ccnf, Mcm4, Gpr132 and Sesn2 changed in opposite ways, being repressed in brain and overexpressed in PBMCs, indicating that their expression is regulated in a cell type-dependent manner.

To confirm the validity of the RT^{2}Profiler™ PCR Array, we further studied the expression changes in genes simultaneously altered in brain and lymphocytes of the APP/PS1 mice by quantitative real-time PCR. In addition, we validated the expression of Cdk4, one of the highly altered genes in lymphocytes. Figure 4 shows a comparison in mRNA abundance as determined by these two methods. As shown, the observed changes were always of the same order of magnitude. Quantitative reverse transcription-PCRs in brain showed significant changes in gene expression between control and transgenic mice (Student's *t*-test: Cdk5rap1, $t_6 = 4.440$, $P = 0.004$; Chek1, $t_6 = 2.510$, $P = 0.05$; E2f1, $t_6 = 2.656$, $P = 0.04$; Abl1, $t_5 = 2.861$, $P = 0.03$; Myb, $t_6 = 2.544$, $P = 0.04$; Nek2, $t_6 = 2.345$, $P = 0.05$; Nfact1, $t_6 = 4.084$, $P = 0.006$; Mcm4, $t_6 = 2.507$, $P = 0.04$; Gpr132, $t_6 = 2.679$, $P = 0.03$; Sesn2, $t_6 = 3.474$, $P = 0.01$; Cdkn2a, $t_6 = 2.322$, $P = 0.05$; Cdkn1a, $t_6 = 3.593$, $P = 0.01$). PCR Arrays in brain and lymphocytes as well as quantitative reverse transcription-PCR analyses in lymphocytes were performed with pooled samples and therefore no statistical analysis was performed.

Proliferative response of peripheral blood mononuclear cells from wild-type and amyloid precursor protein/presenilin 1 mice

To investigate whether there were differences in the proliferative activity following mitogen stimulation between PBMCs derived from control and APP/PS1 mice, we determined the rate of DNA synthesis by assessing the incorporation of BrdU into DNA. We performed three independent experiments using pooled PBMCs derived from blood of four control or APP/PS1 mice. The data in Fig. 5 show that the incorporation of BrdU was significantly higher in APP/PS1 cells than in control PBMCs (Student's *t*-test, $t_4 = 3.735$, $P = 0.02$).

Cell cycle events in the amyloid precursor protein/presenilin 1 mouse brain

The APP/PS1 brain tissue was processed for double-label immunohistochemistry with antibodies against calbindin, a differentiated

5 Alteraciones en la expresión de genes relacionados con el ciclo celular en cerebro y linfocitos de un modelo de ratón transgénico de EA (APP/PS1)

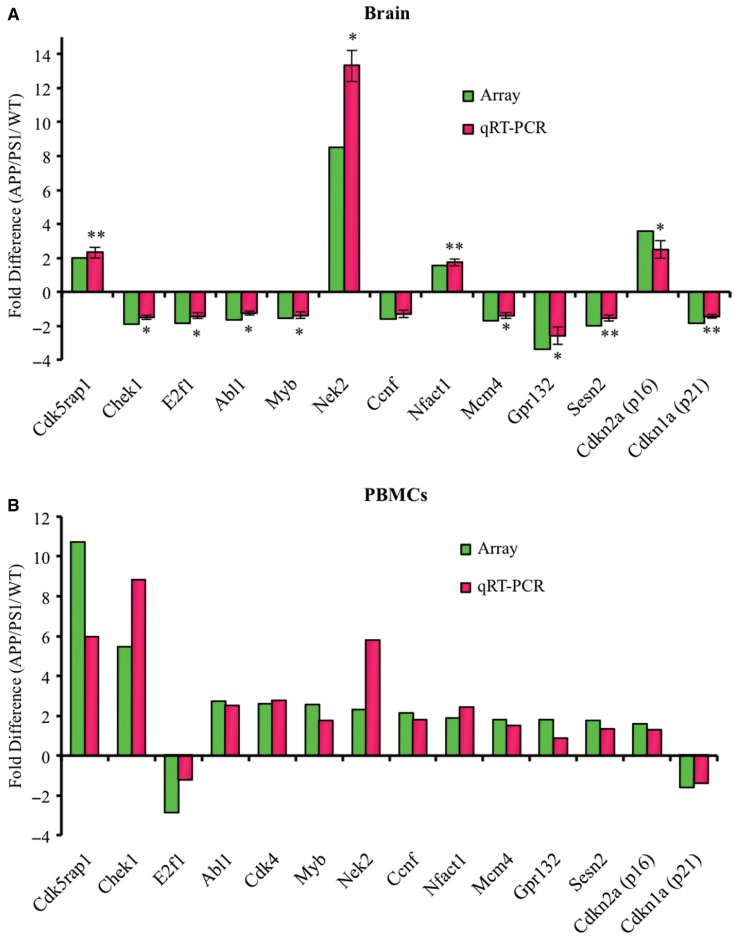

FIG. 4. Quantitative reverse transcription (qRT)-PCR validates changes in cell cycle-related genes in brain (A) and PBMCs (B) from APP/PS1 mice. A comparative ratio analysis of RT^{2}Profiler™ PCR Arrays and qRT-PCR expression profiles of the 13 common altered genes in brain and PBMCs of APP/PS1 mice. In brain, the data for qRT-PCR amplifications represent the mean ± SEM from four different wild-type (WT) or APP/PS1 mice. *$P < 0.05$ significantly different from WT, **$P < 0.01$ significantly different from WT. In PBMCs, data for qRT-PCR were obtained from pooled blood from four WT or APP/PS1 mice.

neuronal marker, and PCNA, a cell cycle marker. Representative photomicrographs of PCNA staining for 12-month-old wild-type and APP/PS1 mice are shown in Fig. 6. The PCNA signal was widely expressed in the granule cell layer but also in the dentate hilus within the hippocampal dentate gyrus of control and APP/PS1 mice (Fig. 6A), accordingly with proliferating hippocampal cells and involved in neurogenesis processes. However, we also observed some neuronal nuclei stained positive for PCNA in the cerebral cortex of APP/PS1 mice (Fig. 6B), a brain region not associated with neurogenesis. In these animals, PCNA-positive nuclei were mainly found in layer II/III, possibly corresponding to small pyramidal cells, in agreement with their shape and size (Fig. 6B). Double staining indicated that an average of ~5% of PCNA-positive cells from APP/PS1 mice co-localized with the neuronal calbindin signal in the cerebral cortex.

We then sought to check whether Cdkn2a or p16, one of the highly altered genes in the APP/PS1 brain, as determined by the PCR Array analysis, was also present in differentiated neurons. Double staining was carried out in the cerebral cortex from wild-type and transgenic mice with anti-p16 antibody and anti-βIII tubulin, a neuronal marker. Moreover, as the cell cycle-related events in the cortex of the APP/PS1 mice could also be ascribed to glial activation, we also performed double staining with anti-glial fibrillary acidic protein (GFAP). Figure 7A shows p16-positive cells mainly in the cortex layer II/III. Co-localization of p16 and βIII tubulin was significantly enhanced in the cortex of the APP/PS1 mice (Fig. 7A). Approximately 20% of the p16 reactivity co-localized with neuronal specific βIII tubulin in the cerebral cortex cells. As expected, activation of the glia was more prominent in the cortex of the APP/PS1 mice (Fig. 7B). Although scarce, we also observed some degree of co-

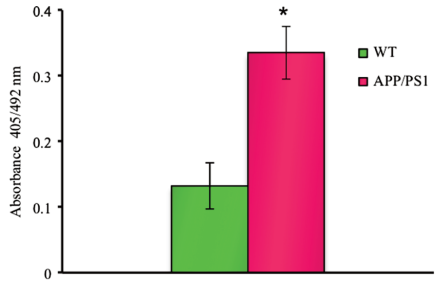

FIG. 5. Proliferative response of wild-type (WT) and APP/PS1 lymphocytes. Lymphocytes obtained from pooled blood from four WT or APP/PS1 mice (5000 cells/well) were seeded in 96-well plates in the presence of mitogens. After 24 h, cells were pulsed with 10 μM BrdU for 4 h. DNA synthesis was assessed by the BrdU incorporation method according to the manufacturer's instructions. Proliferation was expressed as the absorbance of stimulated cultures minus that of non-stimulated cultures. Each bar represents the mean ± SEM of three independent experiments with different pooled blood samples performed in triplicate. *$P < 0.05$ significantly different from WT cells.

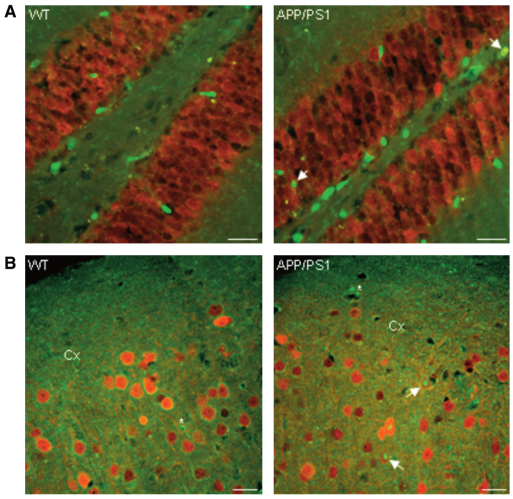

FIG. 6. PCNA expression in brain tissue from wild-type (WT) and APP/PS1 mice. (A) Representative photomicrographs of PCNA (green) and calbindin (red), and their co-localization in the hippocampus, and (B) cortical neurons of 12-month-old WT and APP/PS1 mice. Note scarce PCNA expression (asterisk) in WT and APP/PS1 mouse brain, and that a small amount of the PCNA-positive staining co-localizes with calbindin neurons (arrows), indicating that some differentiated neurons have entered the cell cycle. Scale bar: 20 μm. Cx, cortex.

localization of p16 and glial fibrillary acidic protein only in the cortex of the APP/PS1 mice (Fig. 7B).

Discussion

In this work, we used the RT^{2}Profiler™ PCR Arrays technology to quantify and compare the expression of cell cycle-related genes in brain and PBMCs from a mouse model of AD (APP/PS1) with respect to wild-type mice. To the best of our knowledge, this is the first demonstration of simultaneous cell cycle alteration in both peripheral cells and brain from APP/PS1 mice. In addition, we provide novel experimental evidence for enhanced proliferation of PBMCs from APP/PS1 mice compared with control littermates. It was previously shown that lymphocytes and neuronal cultures from transgenic mice bearing a single or multiple human AD linking pathology PS1 mutations share altered vulnerability to cell stressors (Eckert *et al.*, 2001). Thus, our results add further support to the idea that AD-like pathology induces the systemic derangement of key cellular functions.

Data from our study indicate that the APP/PS1 genotype induced alterations in the expression of a broad spectrum of cell cycle-related genes in both brain and PBMCs. The number of modulated genes was higher in PBMCs than in brain (51 and 23%, respectively). We identified changes in genes associated with many different stages of the cell cycle rather than one specific stage. There were only 13 significantly altered common genes in both tissues. Moreover, some of these genes changed in an opposite manner in these tissues, suggesting that their expression is regulated in a tissue-dependent manner.

Gene expression of Nek2, Cdk5rap1 and Cdkn2a was increased in both brain and PBMCs from the APP/PS1 mice. Nek2 is a member of the serine–threonine kinase family Nek, which is structurally related to the essential mitotic regulator never in mitosis A (Fry *et al.*, 1998). Nek2 is involved in cell division and mitotic regulation by centrosome splitting (Fry, 2002; Fletcher *et al.*, 2004). The Cdk5rap1 gene encodes a protein associated with the regulatory subunit of Cdk5. Cdk5–p25-mediated hyperphosphorylation of tau has been shown to be involved in reactivation of the neuronal cell cycle followed by neuronal death (Hamdane *et al.*, 2005; Hamdane & Buee, 2007). The Cdk inhibitor Cdkn2a or p16 increased by 3.5-fold in brain from APP/PS1 mice, in line with reports indicating the overexpression of p16 in neurons from subjects with AD (Arendt *et al.*, 1996; McShea *et al.*, 1997). Cdkn1a or p21Cip1 was found to be downregulated in both brain and PBMCs of APP/PS1 mice. Altered expression of p21 has also been reported in the frontal cortex of patients with AD (Engidawork *et al.*, 2001; Zhu *et al.*, 2004a). Downregulation of p21 was shown in immortalized lymphocytes from patients with AD, under proliferative conditions (Sala *et al.*, 2008). The expression of the Gpr132 gene is depressed in brain and increased in PBMCs, suggesting distinct functional implications. Gpr132 or G2A receptor was originally identified by virtue of its transcriptional induction in murine B lymphoid cells in response to oncogenic transformation and treatment with various DNA-damaging agents (Weng *et al.*, 1998), and then identified as the receptor for lysophosphatidylcholine (Kabarowski *et al.*, 2001).

In agreement with the observed changes in genes relevant for cell cycle regulation, lymphocytes from APP/PS1 mice showed increased rates of BrdU incorporation into DNA. These observations are in agreement with previous results from our laboratory indicating the enhanced proliferation of PBMCs or immortalized lymphocytes from patients with AD (de las Cuevas *et al.*, 2003; Bartolome *et al.*, 2007; Munoz *et al.*, 2008). However, altered expression of cell cycle-related genes in brain is accompanied by ectopic expression of a proliferation marker such as PCNA and the Cdk inhibitor p16 in post-mitotic neurons, suggesting that APP overexpression and mutated PS1 might push terminally differentiated neurons towards cell cycle re-entry. This finding confirms and extends previous reports indicating increased expression of cell cycle regulatory proteins in brain of a number of murine models of AD (Yang *et al.*, 2006; Ahn *et al.*, 2008; Malik *et al.*, 2008) as well as in patients with AD (Nagy *et al.*, 1997;

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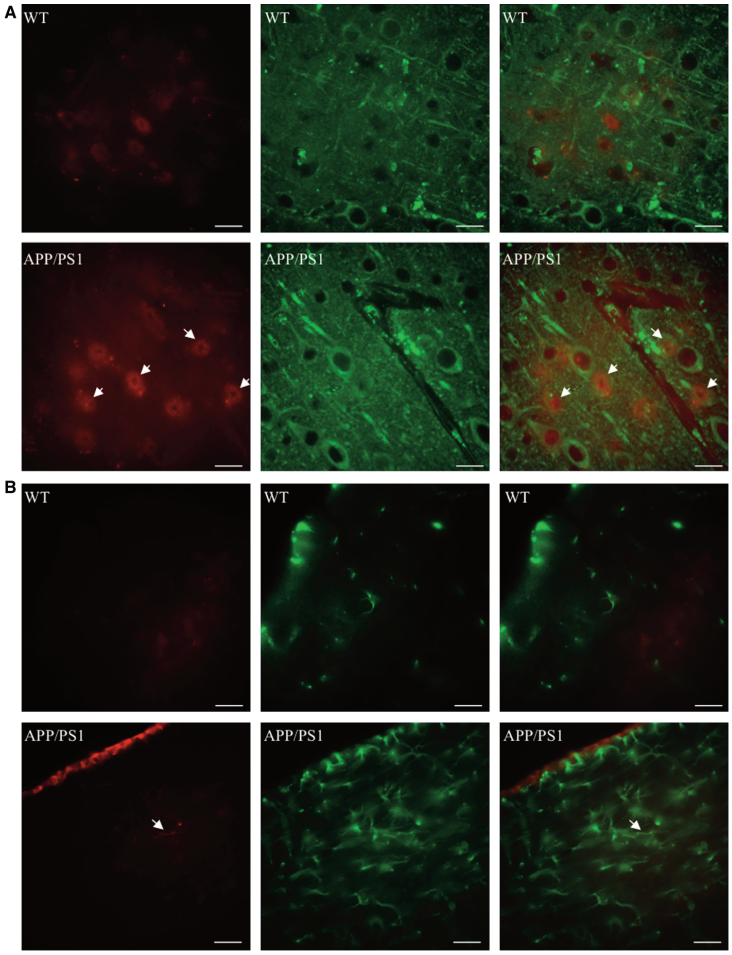

FIG. 7. p16 expression in cortical neurons and astrocytes from wild-type (WT) and APP/PS1 mice. (A) APP/PS1mice (bottom panel) exhibited expression of p16 (red) in βIII tubulin-positive neurons (green) in the frontal cortex layers II/III. This co-localization is absent in WT mice (top panel). Arrows indicate p16-positive neurons, as demonstrated in merged images. (B) Double immunostaining of p16 (red) and glial fibrillary acidic protein (GFAP) (green) shows that few p16-positive cells express the astrocytic phenotype in the frontal cortex layers II/III. Scale bar: 20 μm.

Busser *et al.*, 1998; Schmetsdorf *et al.*, 2007). Nonetheless, it is worth mentioning that other reports showed no evidence of cell cycle reactivation in a triple transgenic mouse model of AD (Lopes *et al.*, 2009).

Despite the evidence for reactivation of the cell cycle in various murine models of AD, and the fact that some apoptotic features were previously shown in the APP/PS1 mice (Spuch *et al.*, 2010), no significant neuronal loss has been reported in any transgenic mice (Oddo *et al.*, 2003; Yang *et al.*, 2006), suggesting that cell cycle disturbances may not be the immediate cause of neuronal death; instead the affected neurons become more vulnerable to other detrimental factors according to the two-hit hypothesis (Zhu *et al.*, 2004b, 2007).

An important limitation of our study is that other cell types, in addition to differentiated neurons, notably neuronal progenitors and glial cells, could contribute to the observed differential cell cycle-related genes in brain tissue from APP/PS1 mice. Although alterations in cell cycle proteins could be demonstrated in the mature neurons within the cerebral cortex of the APP/PS1 mice, it would be desirable to confirm differential gene expression in specific populations of neurons, such as that obtained by laser capture microdissection.

In summary, the results of the present work indicating that cell cycle disturbances occur simultaneously in brain and PBMCs validate the use of peripheral cells from patients with AD as an experimental model to study the cell cycle-induced neurodegeneration in AD. Therefore, either transgenic animals or peripheral cells from patients may be useful to test disease-modifying therapies aiming at avoiding cell cycle re-entry or abrogating cell cycle progression. Drugs sharing the ability to halt cell cycle progression have been shown to provide neuroprotection in murine models of stroke or brain injury (Woods *et al.*, 2007; Varvel *et al.*, 2009), and some prospective studies based on the cell cycle hypothesis are currently ongoing (Bonda *et al.*, 2010).

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Conflict of interest

The authors declare that they have no conflict of interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the article.

Abbreviations

AD, Alzheimer's disease; APP, amyloid precursor protein; BrdU, 5′-bromo-2′-deoxyuridine; CDK, cyclin-dependent kinase; CT, cycle threshold; GFAP, glial fibrillary acidic protein; PBMC, peripheral blood mononuclear cell; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PS1, presenilin 1.

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Evaluación sistemática de las técnicas de Resonancia Magnética de Imagen y Espectroscopía para la caracterización del modelo transgénico de alzhéimer APP/PS1

6

Artículo #5 : **Noemí Esteras**, Carolina Alquézar, Fernando Bartolomé, Desiré Antequera, Laura Barrios, Eva Carro, Sebastián Cerdán, Ángeles Martín-Requero (2012). "Systematic Evaluation of Magnetic Resonance Imaging and Spectroscopy Techniques for Imaging a Transgenic Model of Alzheimer's Disease (AbetaPP/PS1)." **J Alzheimers Dis** 30(2): 337-353.

Resumen

Los modelos murinos de la enfermedad de Alzheimer proporcionan una herramienta muy útil para la búsqueda y seguimiento de biomarcadores de la enfermedad similares a los que se observan en humanos. Las técnicas no invasivas, como la Resonancia Magnética de Imagen (MRI) y de Espectroscopía (MRS) son muy convenientes para estos propósitos, ya que permiten estudiar *in vivo* la morfología y el metabolismo cerebral en cualquier momento del curso de la enfermedad. En este trabajo hemos empleado ratones transgénicos APP/PS1 y ratones control de 12 meses de edad en los que hemos estudiado sistemáticamente los parámetros que estas técnicas nos proporcionan. En primer lugar, los estudios de MRI nos permitieron obtener una colección de imágenes pesadas en T2, en las que pudimos estudiar la morfología del cerebro y de las distintas estructuras que lo forman, así como imágenes de difusión y de transferencia de magnetización, que nos permitieron evaluar la integridad y la estructura macromolecular del tejido. Comparados con los ratones control, los ratones transgénicos muestran hiperintensidad en la corteza cerebral, un aumento del volumen ventricular, así como una disminución del volumen del hipocampo y de la transferencia de magnetización. Los estudios de MRS, por su parte, nos permitieron evaluar distintos metabolitos en las áreas cortical y subcortical del cerebro de los ratones. El análisis revela un incremento en el cociente colina (Cho) / creatina (Cr) en ambas áreas. El tratamiento estadístico de todos los parámetros por regresión logística nos permitió identificar aquellos que permitían clasificar con mayor sensibilidad y especificidad a los ratones en control o transgénico, encontrando que por sí solo, el cociente Cho/Cr en el área subcortical permite la identificación correcta del ratón. En resumen, estos resultados permiten evaluar los marcadores MRI/MRS óptimos para la caracterización de la EA en el modelo APP/PS1, que podrían aplicarse para el diagnóstico, el estudio de la progresión de la enfermedad o el diseño de nuevos fármacos, dada la naturaleza no invasiva de la tecnología.

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Systematic Evaluation of Magnetic Resonance Imaging and Spectroscopy Techniques for Imaging a Transgenic Model of Alzheimer's Disease (AβPP/PS1)

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Abstract. Murine models of Alzheimer's disease (AD) provide means to detect and follow biomarker changes similar to those observed in humans. Non-invasive biomarkers, such as those provided by magnetic resonance imaging (MRI) and spectroscopy (MRS) methods are highly desirable, however, systematic studies of *in vivo* MRI/MRS methods to characterize the cerebral morphology and metabolic pattern of these mice remain scarce. We investigated sixteen consecutive slices from the brain of wild-type and AβPP/PS1 mice, obtaining a collection of T_2-weighted, diffusion weighted and magnetization transfer weighted images as well as ^{1}H PRESS spectra from the cortical and subcortical areas. Compared to controls, AβPP/PS1 mice show significant regional hyperintensities in T_2-weighted images of the cerebral cortex, significant ventricular enlargement, and decreased hippocampal area and fractional magnetization transfer. MRS demonstrated an increase in the ratio of choline (Cho) to creatine (Cr) in the cortical and subcortical areas of the transgenic animals. A logistic regression classifier was implemented considering all parameters investigated, and revealed the most characteristic changes and allowed for the correct classification of control and AβPP/PS1 mice. In summary, the present results provide a useful frame to evaluate optimal MRI/MRS biomarkers for the characterization of AD models, potentially applicable in drug discovery processes, because of their non-invasive and repeatable nature in longitudinal studies.

Keywords: ADC maps, Alzheimer's disease, MRI, MRS, MT maps, T_2-weighting, transgenic mice, ventricle enlargement

INTRODUCTION

Alzheimer's disease (AD) is a devastating neurodegenerative disorder affecting more than 20 million people worldwide [1]. Key histopathological features include amyloid-β (Aβ)-containing senile plaques and neurofibrillary tangles (NFTs), along with neuronal loss in selected brain areas [2]. Presently, a definitive diagnosis of AD is possible only by examining brain tissue after death and requires anatomical evidence for the existence of plaques and NFTs. However, advances in clinical diagnostic tools, including brain imaging

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techniques and novel biomarkers, could potentially help identify 'at risk' subjects prior to the presence of any cognitive deterioration. Moreover, imaging techniques and/or specific biomarkers for AD hold great promise concerning monitoring disease status and treatment response.

A small proportion of AD patients expresses mutations in the amyloid-β precursor protein (AβPP) and presenilin (PS1 and PS2) proteins or overexpress AβPP itself [3]. These 'familial' AD patients suffer from earlier and more rapid, but otherwise identical, amyloid deposition, brain atrophy, and cognitive decline than late-onset AD patients, who form the majority of cases. These human mutations have provided the means to generate a number of transgenic mice differing in the number and type of AβPP and PS mutations expressed, as well as in the promoters that govern transgene expression (for reviews, see [4, 5]). Although none of these models fully replicate the human disease, they have provided valuable insights into disease mechanisms [6]. Therefore, it becomes relevant to apply technologies and biomarkers similar to those used in humans for the follow-up of AD animal models, particularly for the clarification of mechanisms and for the screening and validation of new candidate treatments.

Magnetic resonance imaging (MRI) and spectroscopy (MRS) methodologies provide comprehensive and non-invasive information of cerebral morphology and metabolism. In particular, MRI has been most widely used in AD research to visualize the onset and advance of neurodegeneration, with the goal of identifying particular brain regions that can predict the likelihood of progressing from mild cognitive impairment (MCI) to dementia [7–12]. MR technologies have also been used in normal and genetically engineered mice [13–17]. However, many of these studies have relied in the segmentation of preselected regions-of-interest (ROI), known to become severely degenerated in AD [18], neglecting the potential of the MR method to provide more comprehensive information on the whole brain. Moreover, no systematic assessment of the relative efficacy of different MRI/MRS methods, including anatomical T_2-weighted, diffusion weighted, magnetization transfer (MT) imaging or the ^{1}H spectroscopy methods in the evaluation of AD is currently available.

On these grounds, we present here a study combining a variety of *in vivo* MRI/MRS measurements through the complete brain of AβPP/PS1 mice and wild-type littermates, to evaluate the relative potential of each approach in the characterization of the AD phenotype. To this end, we have implemented a logistic regression algorithm to identify the MRI/MRS parameter or combination of parameters that best discriminate between controls and a transgenic mouse model of AD. Moreover, since previous studies [19] indicated that *in vivo* imaging can predict levels of neurogenesis in the living mice, we investigated whether anatomical changes in the hippocampus could compromise the proliferation of neuronal precursors in this region. For the present studies, we chose AβPP/PS1 mice as the transgenic model of AD. These mice are a cross of the Tg2576 (over-expressing human AβPP (hAβPP) 695, with the double mutation KM670/671NL), and mutant PS1 (M146 L) mice. This murine model of AD typically expresses sufficiently high levels of hAβPP and Aβ to insure amyloid deposition [20]. Aβ is deposited progressively in the brain of these mice beginning at eight weeks of age [21], associated with extensive dystrophy of neurites. In addition, these mice, carrying mutations in the two AD proteins, depict signs of apoptotic cell death in the hippocampus and cerebral cortex of six-nine month-old animals [22, 23].

We report that AβPP/PS1 mice depict enlarged ventricles and decreased hippocampal volume, together with decreased MT, enhanced neurogenesis, and increases in the choline (Cho) signal in the spectra of the cerebral cortex and subcortical area. Taken together, our results indicate that the Cho/creatine (Cr) ratio, as measured in the subcortical region provides an optimal biomarker of disease status, fully discriminating between control and AβPP/PS1 mice that are twelve months of age. In addition, our results suggest that MRI/MRS technologies provide a robust and convenient tool for the evaluation of AD disease status and disease-modifying therapies in the AβPP/PS1 mouse model.

METHODS

Animal model and experimental design

All procedures with animals were specifically approved by the 'Ethics Committee for Animal Experimentation' of the Center for Biological Research (CIB, CSIC), and carried out in accordance with the protocols issued which followed National (normative 1201/2005) and International recommendations (normative 86/609 from the European Communities Council). Adequate measures were taken to minimize pain or discomfort of animals. Double-transgenic AβPP/PS1 male mice ($n = 7$, 12 months of age), a

cross of the Tg2576 (overexpressing human-AβPP 695 with double mutations at KM670/671NL) and mutant PS1 (M146L) mice, based on the C57BJ6 phenotype, were used as a model of AD amyloidosis [24]. Non-transgenic C57BJ6 male littermates ($n = 8$) were used as controls.

Preanesthesia was induced, before MR examinations, by administering a mixture of 97%/3% oxygen/isofluorane with the animal placed in the interior of a plexiglass chamber. The animal was then positioned in the NMR cradle, transferred to the center of the magnetic field and the anesthesia maintained throughout the imaging procedure by administering a mixture of 98%/2% oxygen/isofluorane (1 mL/min) through a nose-cap. Animal temperature was maintained at 37°C with a water heated pad adapted to the cradle. The respiratory rate of the animals was monitored using a Biotrig physiological monitor (Bruker Medical Gmbh, Ettlingen, Germany).

MRI/MRS methods

The MRI experiments were performed on a Bruker Pharmascan 7.0-T 16 cm horizontal-bore system (Bruker Medical Gmbh, Ettlingen, Germany), equipped with a 23 mm mouse head ^{1}H selective birdcage resonator and a 90 mm diameter gradient insert (maximum intensity 30 G/cm). All data were acquired using a Hewlett-Packard console running Paravision software v 5.1 operating under a Linux environment.

T_2-weighted (T_2-W) spin-echo anatomical images used a rapid acquisition method with relaxation enhancement (RARE) in axial orientations with the following parameters: TR = 2500 ms, TE = 60 ms, RARE factor = 8, Av = 6, FOV = 2.3 cm, acquisition matrix = 256 × 256, slice thickness = 1.00 mm and number of slices = 16. Image Processing was performed with the ImageJ Package (Wayne Rasband, National Institutes of Health, USA, http://rsbweb.nih.gov/ij/). Ventricular and hippocampus areas determined in each T_2-weighted slice were normalized to the corresponding total brain area, and compared in control and AβPP/PS1 mice. Apparent diffusion coefficient (ADC) maps were obtained from diffusion weighted images acquired with increasing b values, essentially as previously described [25]. The following parameters were used: TR = 3000 ms, TE = 51 ms, Av = 3, diffusion gradient duration = 4 ms, diffusion gradient separation = 16 ms, diffusion gradient direction: left-right, FOV: 3.8 cm, acquisition matrix = 128 × 128, b values = 100, 200, 300, 500, 800, and 1200 s/mm^2. The ADC maps were calculated by fitting non-linearly the function $I_b = I_o\, e^{-ADC.b}$ to every pixel where: I_b = signal intensity at value b, I_o = Signal intensity for b = o, b = diffusion weighting factor and ADC = apparent diffusion coefficient.

MT maps were acquired with a multi-slice multi-echo sequence in axial orientation and the following parameters: TR = 2500 ms, TE = 9.8 ms, Av = 1, FOV = 2.0 cm, acquisition matrix = 128 × 128, after applying (Mt) or not (Mo) a magnetization transfer saturation pulse at 8.0 ppm. M(t)/M(0) maps were prepared by dividing pixel by pixel the intensities of saturated and non-saturated images.

Pixel by pixel operations and non-linear fittings of diffusion data sets were performed using the home-made software My Map Analyzer, running under MatLab v7 in a LINUX based platform.

Images were registered and aligned using SPM software (Neuroimaging Informatics Tools and Resources Clearinghouse, USA, http://www.nitrc.org/projects/spm/). The cerebral regions of interest (cortex, hippocampus, ventricles, etc.) were then delineated by superimposing the anatomical information from a brain atlas [26] on the MR image or map. The internal anatomical mark provided by the dorsal third ventricle (Bregma −1.46 mm, interaural 2.34 mm), was used as a common reference between the image collection and the atlas, to obtain an adequate anatomical localization of the MR sections observed in every animal. ROIs were selected with the Image J software. *In vivo* ^{1}H MR spectroscopy used a point-resolved spatially spectroscopy sequence (PRESS), combined with VAPOR water suppression with the following parameters: TR = 3000 ms, TE = 35 ms, Av = 128, voxel volume = 3 mm^3. Clinically detectable metabolites as N-acetylaspartic acid (NAA), total Cr, and total Cho, were quantified by measuring the area of the peaks using the LC Model software [27]. These were in all cases below the 20% Cramér–Rao lower bound (CRLBs), an estimate of the standard deviation (SD) of the fit for each metabolite. Additional metabolites investigated by LCModel as lactate, glutamate, glutamine, myo-inositol, and taurine did not overcome the CRLBs constraints and were not further considered in the analysis.

Immunohistochemical methods

5-Bromodeoxyuridine (BrdU) (Sigma, St Louis, MO), dissolved in 0.9% NaCl at dose of 50 mg/kg, was administered intraperitoneally (i.p.) for seven consecutive days, and the mice then sacrificed one day after the last injection. Animals were perfused

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transcardially with saline buffer or 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Fixed brains were cut on a vibratome (Leica Microsystems) at 50 μm, and tissue sections were collected in cold PB 0.1 M. DNA was denatured by incubating tissue sections for 30 min in 2 N HCl at room temperature. Then, sections were blocked by incubating for 15 min in a solution containing 10% methanol and 3% hydrogen peroxide, and incubated overnight with a mouse monoclonal anti-BrdU antibody (1 : 20000, DS Hybridoma Bank). For stereological estimation of $BrdU^+$ cells, all sections were processed for single BrdU immunohistochemistry using the avidin-biotin complex method (VECTASTAIN Elite ABC Kit, Vector Laboratories, Burlingame, CA). The reaction was visualized using 3,3′-diaminobenzidine (Vector Laboratories) as the chromogen. The brain was sectioned at 50 μm, and every sixth section, spaced 300 μm apart throughout the entire rostral/caudal extent of the hippocampus (from Bregma −1.06 mm to Bregma –3.80 mm), was used to assess the number of BrdU-labeled cells. In addition, brain sections were also incubated with a goat polyclonal doublecortin antibody (DCX; 1 : 250, Santa Cruz Biotechnology, Inc.) to identify neuronal progenitor cells, and with a rabbit polyclonal anti-Ki-67 antibody (1 : 500, Abcam, Cambridge, UK), a useful marker for cellular proliferation. Secondary antibodies were: donkey anti-goat IgG 488 (1 : 1000, FluoProbes®, Interchim), and Texas Red goat anti-rabbit IgG antibody (Jackson Immunoresearch). These sections were co-labeled with DAPI nuclear staining (Sigma-Aldrich). The same areas and number of sections were studied in all animals and experimental groups. The specificity of the staining was tested by omitting primary antibodies from the incubation solution. Images were captured using a Zeiss LSM 510 Meta scanning laser confocal microscope (Carl Zeiss Microimaging, GmbH).

Statistics

Statistical analysis was performed using the SPSS package (Version 18.0) for Windows. Levene's test was run to analyze the homogeneity of variance between wild-type mice and transgenic AβPP/PS1 mice. All statistical tests were two-tailed *t*-test with significance set at the 0.05 level. Logistic regression analyses were performed with the MRS data in cortical and subcortical areas and with the MRI measurements in the whole brain, cortex and hippocampus [28]. To identify the independent predictors of transgenesis, the measurement from each region with the highest score and significant difference was selected and combined with other variables in a stepwise analysis. The posterior probability was calculated with the formula: $P(x) = 1/1 + e^{-(\beta_o + \beta_1 x_1 + \beta_2 x_2 + .. \beta_t x_t)}$, where x_1, $x_2 \ldots x_t$ represent independent variables. The probability of reference for classification was 0.5.

RESULTS

Figure 1A illustrates the anatomical location of the sixteen consecutive slices acquired by MRI increasing from the caudal to the rostral planes, respectively. Briefly, we used the dorsal third ventricle as an internal anatomical marker from control and AβPP/PS1 mice to align, register, and compare the collection of images from each mouse. The same internal anatomical coordinates allowed the selection of the corresponding sections of the atlas that best described those obtained by MRI. Normally, the dorsal third ventricle was contained in slice 8, providing a robust anatomical coordinate for the localization of the remaining slices. Table 1 summarizes the main anatomical regions contained in each one of the sixteen slices. In this way, the entire collection of MRI images and anatomical maps could be aligned, regionalized, and analyzed. The right insert shows a representative example of the anatomical regionalization of slice 4, as obtained by superimposing the corresponding region from the anatomical atlas over the MRI image. We have considered three main regions in each slice: (i) the whole brain section, including all the anatomical structures therein (Table 1); (ii) the cerebral cortex; and (iii) the hippocampus, as regionalized in the MRI image by superimposing the atlas. Figure 1B depicts consecutive T_2-weighted MR sections (slices 4–10) through the brain of control (upper panels) and AβPP/PS1 mice (lower panels). The bar graphs shown below quantitatively compare the average intensity (±standard error) of the three selected regions in each slice from control (white bars) and AβPP/PS1 (black bars). The average T_2-w intensity is similar in all slices of the whole brain, presenting a tendency to be higher in the AβPP/PS1 mice in the cortex and hippocampus, with statistically significant values ($p < 0.05$) in the cortex from slices 9 and 10, primarily involved in the control of motor activities.

Figure 2 provides a quantitative comparison of the areas occupied by the ventricles (solid perimeter), and the hippocampus (broken perimeter) in different slices of control (white bars) and AβPP/PS1 mice (black bars), as determined in the same T_2-weighted image

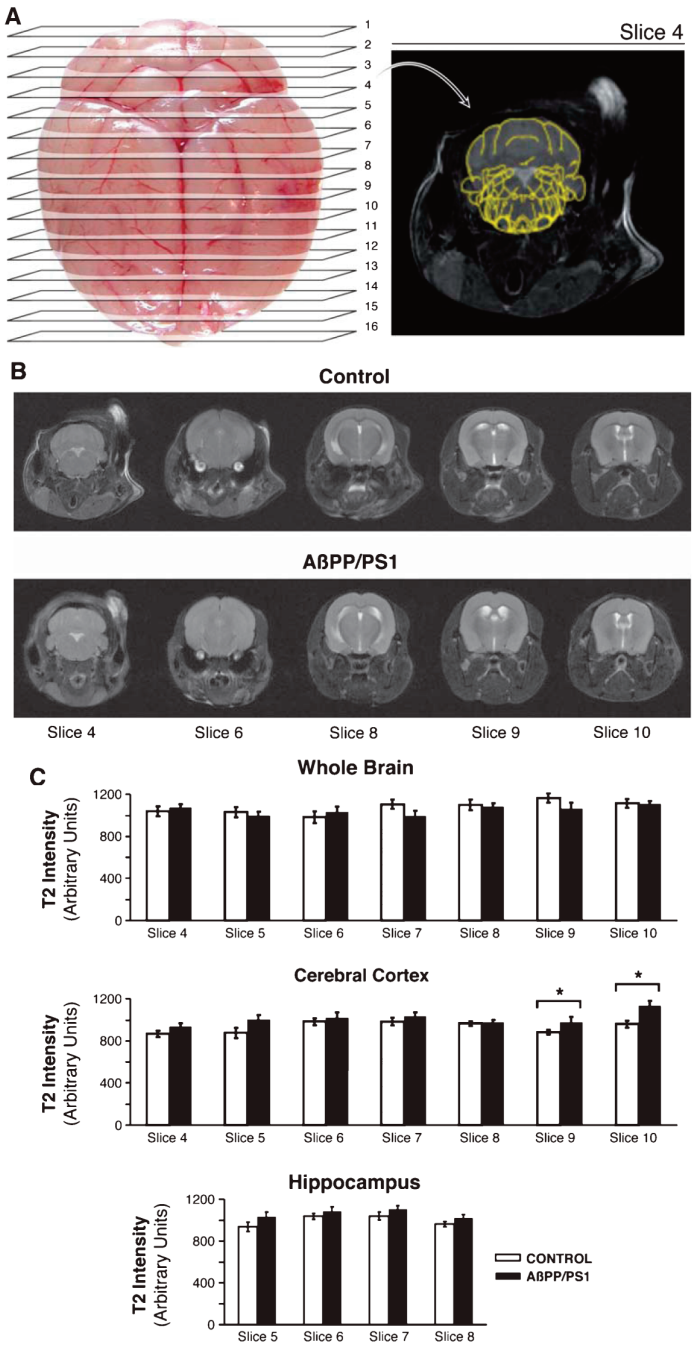

Fig. 1. *In vivo* T_2-weighted brain images of wild-type and AβPP/PS1 mice. A) Sixteen consecutive slices (1 mm thick), with increasing numbering in the caudal to rostral direction were acquired for each mouse (left panel). Regionalization was performed using the dorsal third ventricle as a common anatomical reference between the cerebral image and the Paxinus atlas (right panel). B) T_2-weighted images (slices 4–10) through the coronal plane of control and AβPP/PS1 mice. C) Relative intensity in the whole brain, cerebral cortex and hippocampus as determined in each slice using the ImageJ software. Values shown are the mean ± standard error (control mice $n = 8$; Tg mice $n = 7$). *$p < 0.05$.

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Table 1
Main anatomical structures found in each T_2 slice, as determined in the anatomical atlas. Percent values indicated for the corresponding anatomical structures indicate the relative area of the structure(s) as compared to that of the complete cerebral section, as described in [26]

Slice	Main anatomical structures
Slice 2	Cerebellum 65%
	Hindbrain 35%
Slice 3	Cerebellum 60%
	Hindbrain 38%
	Ventricles – Fourth ventricle 2%
Slice 4	Cerebellum 60%
	Hindbrain (Medulla and pons) 33%
	Ventricles – Fourth ventricle 7%
Slice 5	Cerebral cortex 55%
	Midbrain 25%
	Hindbrain (Pons and medulla) 20%
Slice 6	Midbrain 37%
	Cerebral cortex 35%
	Hippocampus 25%
	Thalamus 3%
Slice 7	Cerebral cortex 40%
	Hippocampus 15%
	Ventricles 15%
	Hypothalamus 12%
	Thalamus 12%
	Midbrain 6%
Slice 8	Cerebral cortex 54%
	Thalamus 20%
	Cerebral nuclei (Striatum, Pallidum) 8%
	Hypothalamus 8%
	Ventricles 6%
	Hippocampus 4%
Slice 9	Cerebral cortex 45%
	Cerebral nuclei (Striatum, Pallidum) 25%
	Ventricles 10%
	Thalamus 9%
	Hypothalamus 9%
	Hippocampus 2%
Slice 10	Cerebral cortex 45%
	Cerebral nuclei (Striatum, Pallidum) 35%
	Ventricles 10%
	Hypothalamus 5%
	Thalamus 5%
Slice 11	Cerebral cortex 54%
	Nuclei 40%
	Hypothalamus 3%
	Ventricles 3%
Slice 12	Cerebral cortex 70%
	Cerebral nuclei 30%
Slice 13	Cerebral cortex 100%

data set shown in Fig. 1. The relative ventricular area is significantly larger ($*p < 0.05$) in slices 8–10, while the relative hippocampal area decreases significantly ($*p < 0.05$) in slice 9 of the AβPP/PS1 mice (Fig. 2B, C). A more detailed analysis of the areas corresponding to the left and right lateral ventricles, the third ventricle, and the third dorsal ventricle showed that the differences are found mainly between the areas of the left and right lateral ventricles (Fig. 2D). When we calculate the corresponding ventricular and hippocampal relative volumes (Fig. 2E, F) by multiplying the measured areas by the thickness of the MRI sections, the ventricular volumes are appreciably larger while the hippocampal volumes remained smaller in the AβPP/PS1 mice.

To investigate the possibilities that these increases in ventricular volume of AβPP/PS1 mice would involve edema of the cerebral parenchyma, we have studied the effects of the AβPP/PS1 genotype on the MT and ADC Maps. In these cases, we acquired only five slices through brain; slice 4 in these acquisitions corresponds essentially to slice 9 of the former series, maintaining the same coordinate system. The main anatomical structures included in these slices are summarized in Table 2. Fig. 3 compares MT maps, obtained as indicated in the Methods, from the whole brain, cerebral cortex, and hippocampus from representative slices (1–5) of control and AβPP/PS1 mice. The AβPP/PS1 genotype results in a decrease of relative MT in all structures investigated. The decrease becomes statistically significant ($*p < 0.05$) in whole brain (slices 3 and 5), cerebral cortex (slices 3–5), and hippocampus (slice 3).

To confirm this hypothesis, we obtained ADC maps from the same slices (1–5) of control and AβPP/PS1 mice (Fig. 4). ADC maps show a tendency to increase in the three regions, with more apparent increases in slices 2 and 4 of the whole brain and cerebral cortex and slice 2 of the hippocampus. These results reveal a relative increase of the ADC consistent with an increased ventricular volume and intracellular water content or decreased averaged obstructions to water translation. Together, the MT and ADC maps obtained are consistent with a generalized cerebral edema in the brain from AβPP/PS1 mice.

We further investigated the effects of the AβPP/PS1 genotype on the ^{1}H MRS spectra obtained from the cortex (Fig. 5A, B) and subcortical areas (Fig. 5D, E). Figure 5 shows representative results from ^{1}H MRS spectra obtained from these regions from control (Fig. 5A, D) and AβPP/PS1 mice (Fig. 5B, E). A quantitative analysis of the relative intensities of the NAA, total Cr, and total Cho resonances, the metabolites most commonly detected in clinical scanners, is shown in the bar graphs of Fig. 5C, F. While the NAA/Cr does not change appreciably between control and AβPP/PS1 mice, the Cho/Cr ratio increases significantly ($*p < 0.005$) in the cortical and subcortical areas of the AβPP/PS1 mice. It should be noted here that the Cho peak is a composite resonance containing unresolvable contributions *in vivo* from choline,

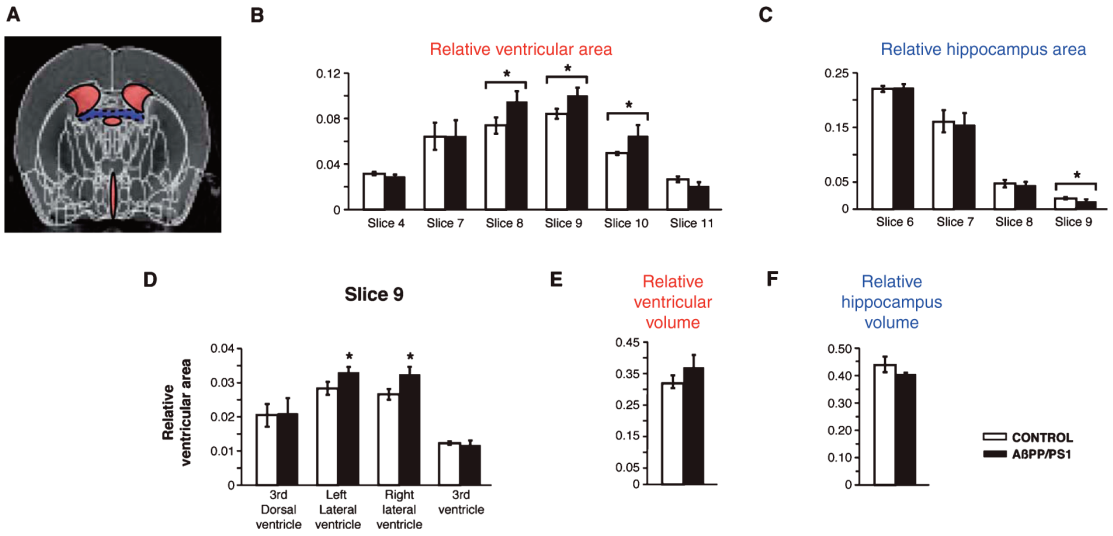

Fig. 2. Comparison between ventricular and hippocampus size in wild-type and AβPP/PS1 mice. A) Regions used to determine the ventricular area (solid line) and hippocampus area (broken line). B, C) Ventricular and hippocampus areas are expressed relatively to the total brain area of each slice in control (white bars) and AβPP/PS1 (black bars) mice. Values are the mean ± standard error (control mice $n=8$; Tg mice $n=7$). *$p<0.05$. D) Ventricular enlargement occurs primarily in the lateral ventricles. E, F) Ventricular or hippocampus volumes were determined from the addition of the relative volumes of these structures in the corresponding slices, calculated as the product of their relative area by slice thickness.

Table 2
Main anatomical structures observed in each MT and ADC slice, as determined in the anatomical atlas. Percent values indicated for the corresponding anatomical structures indicate the relative area of the structure as compared to that of the complete cerebral section, as described in [26]

Slice	Main anatomical structures
Slice 1	Midbrain 37%
(Slice 6 in T_2)	Cerebral cortex 35%
	Hippocampus 25%
	Thalamus 3%
Slice 2	Cerebral cortex 40%
(Slice 7 in T_2)	Hippocampus 15%
	Ventricles 15%
	Hypothalamus 12%
	Thalamus 12%
	Midbrain 6%
Slice 3	Cerebral cortex 45%
(Slice 9 in T_2)	Cerebral nuclei (Striatum, Pallidum) 25%
	Ventricles 10%
	Thalamus 9%
	Hypothalamus 9%
	Hippocampus 2%
Slice 4	Cerebral cortex 45%
(Slice 10 in T_2)	Cerebral nuclei (Striatum, Pallidum) 35%
	Ventricles 10%
	Hypothalamus 5%
	Thalamus 5%
Slice 5	Cerebral cortex 54%
(Slice 11 in T_2)	Nuclei 40%
	Hypothalamus 3%
	Ventricles 3%

phosphorylcholine, and glycerolphosphorylcholine. Under these circumstances the increases observed could be due to augmented concentrations of one or more of these metabolites.

Proliferation of neuronal progenitors is enhanced in AβPP/PS1 mice

High MRS Cho signals are accepted predictors of accelerated membranous turnover and proliferative activity. Although glial proliferation may be expected in the transgenic mice, as it was reported in AD brain [29], we found it interesting to elucidate whether neurogenesis could be perturbed in the AβPP/PS1 mice. For these experiments, mice were injected with BrdU for seven days prior the determination of BrdU incorporation by immunohistochemistry. Figure 6 shows that the number of $BrdU^+$ cells in the hippocampus, including dentate gyrus (DG), CA1, and CA3 is significantly higher (*$p<0.05$) in the transgenic mice than in the wild-type littermate mice. Proliferation of neuronal progenitors in the hippocampus of control and AβPP/PS1 mice was also labeled by Ki-67 and DCX staining. Representative photomicrographs of Ki-67 and DCX staining are shown in Fig. 7A, B, respectively.

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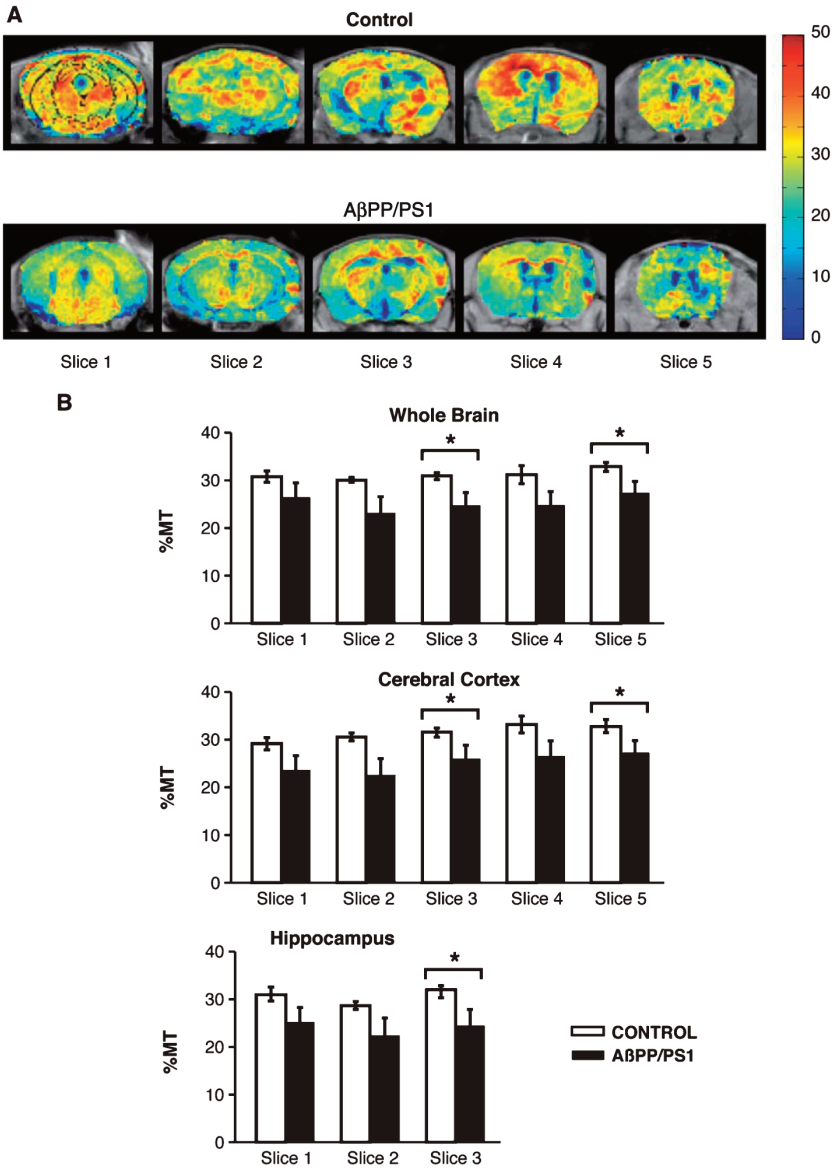

Fig. 3. Comparison between MT Maps from wild-type and AβPP/PS1 mice. A) % MT maps in slices 1–5 of control (top) and AβPP/PS1 mice. Note the decrease in % MT in AβPP/PS1. B) Regional % MT maps in whole brain (top), cerebral cortex (center) and hippocampus (bottom) of control (white bars) and AβPP/PS1 (black bars) mice. Values shown represent the mean ± standard error (control mice $n = 8$; Tg mice $n = 7$). *$p < 0.05$.

Logistic regression analyses of MR parameters

Logistic regression analyses were performed for MRI data in whole brain, cerebral cortex, and hippocampus, as well as for the MRS data in the cortical and subcortical areas, to identify within these different biomarkers those that best discriminate between the control and AβPP/PS1 groups. For each region, the measurement with the highest score and significant difference was selected and combined sequentially and hierarchically with the remaining variables as indicated in the legend (Fig. 8).

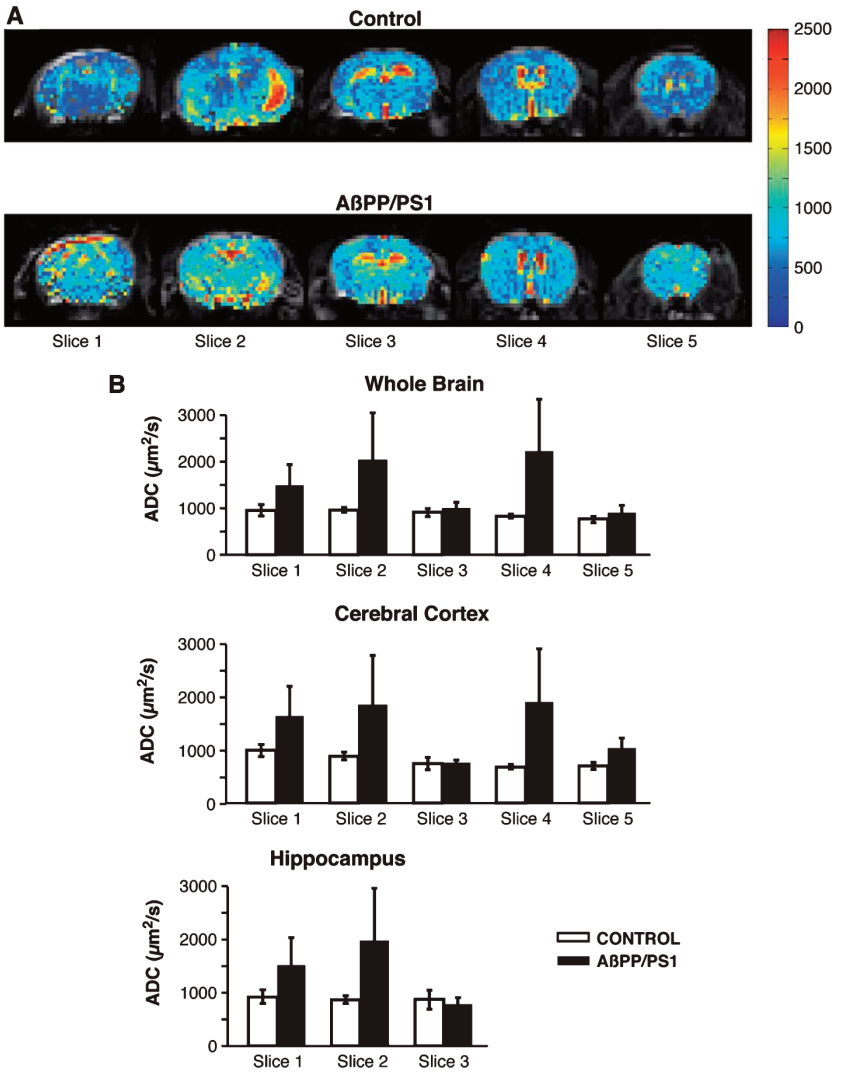

Fig. 4. Comparison between ADC maps from wild-type and AβPP/PS1 mice. A) ADC maps in slices 1–5 of Control (top) and AβPP/PS1 mice. Note the average increase in the color scale ADC in AβPP/PS1. B) Regional ADC maps in whole brain (top), cerebral cortex (center) and hippocampus (bottom) of control (white bars) and AβPP/PS1 (black bars) mice. Values shown represent the mean ± standard error (control mice $n = 8$; Tg mice $n = 7$).

Figure 8A summarizes the individual posterior probability of being correctly classified as control (open symbols) or transgenic mice (filled symbols), while Fig. 8B depicts the percentages of sensitivity/specificity of the classifier, found by using different combinations of MRI or MRS parameters. These values are 86/87 when combining T_2 signal in slice 9 and 4 in the whole brain (see Table 1 for main anatomical regions involved). The same values were obtained in the cerebral cortex with the T_2 signal in slice 10 plus the % of MT in slice 2, and reached the 100% classification success when the ADC value in slice 1 is included. MRI parameters in the hippocampus had no statistically significant discriminatory value. Interestingly, the ventricular or the hippocampal areas provided relatively poor accuracy in distinguishing AβPP/PS1 from control mice (72% and 66% respectively). Analysis of the MRS data indicates that the ratio of Cho/Cr

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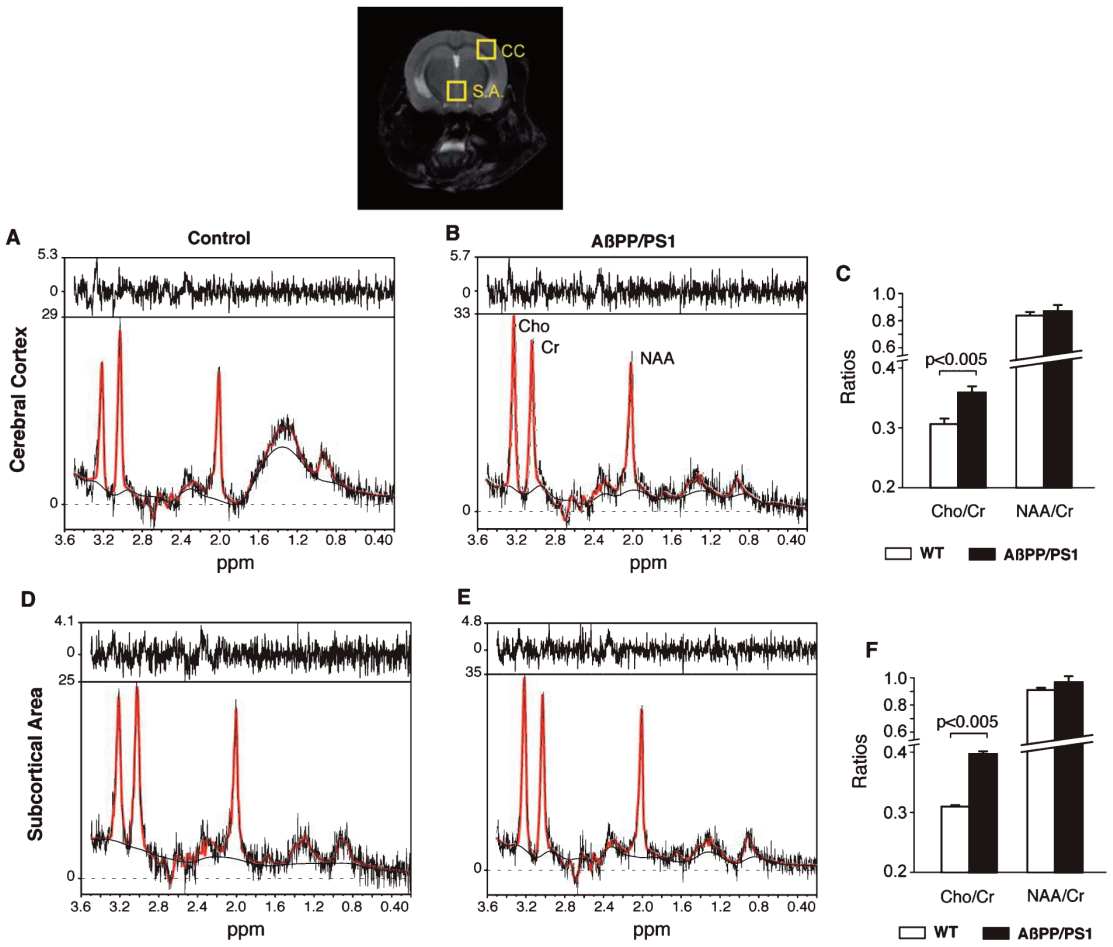

Fig. 5. *In vivo* ^{1}H PRESS spectra from cortical and subcortical areas of wild-type and AβPP/PS1 mice. Top insert: Cortical and subcortical voxel localization (yellow squares). Representative spectra form cortical (A,B) and subcortical (D,E) areas of control and AβPP/PS1 mice, respectively. Cho/Cr and NAA/Cr ratios in the cortical (C) and subcortical (F) regions of control (white bars) and AβPP/PS1 (black bars) mice. Note the significant increase in Cho/Cr ratio in the AβPP/PS1 mice. Values shown are the mean ± standard error (control mice $n = 8$; Tg mice $n = 7$).

in the subcortical area is the best predictor, since it completely and precisely discriminates between control and transgenic mice, just in the first step. It is worthwhile mentioning here that the same ratio Cho/Cr in the cortex provides lower scores, highlighting the importance of the region selection procedure in the diagnostic process.

DISCUSSION

The search for homologous biomarkers of disease for humans and animals represents an important step in the drug discovery process. When found, these may be applied longitudinally both during preclinical research and in the clinical trials, in a fully translational environment during therapy evaluation. AD biomarkers are biochemical and anatomical variables that measure AD-related pathological features. Biomarkers are either utilized for early diagnosis in asymptomatic individuals or as end-point biomarkers in symptomatic subjects or in disease-modifying therapies. Non-invasive biomarkers, such as those provided by MRI and MRS are endowed with optimal properties for these purposes, mainly because their non-invasive character and the applications of the same MRI/MRS

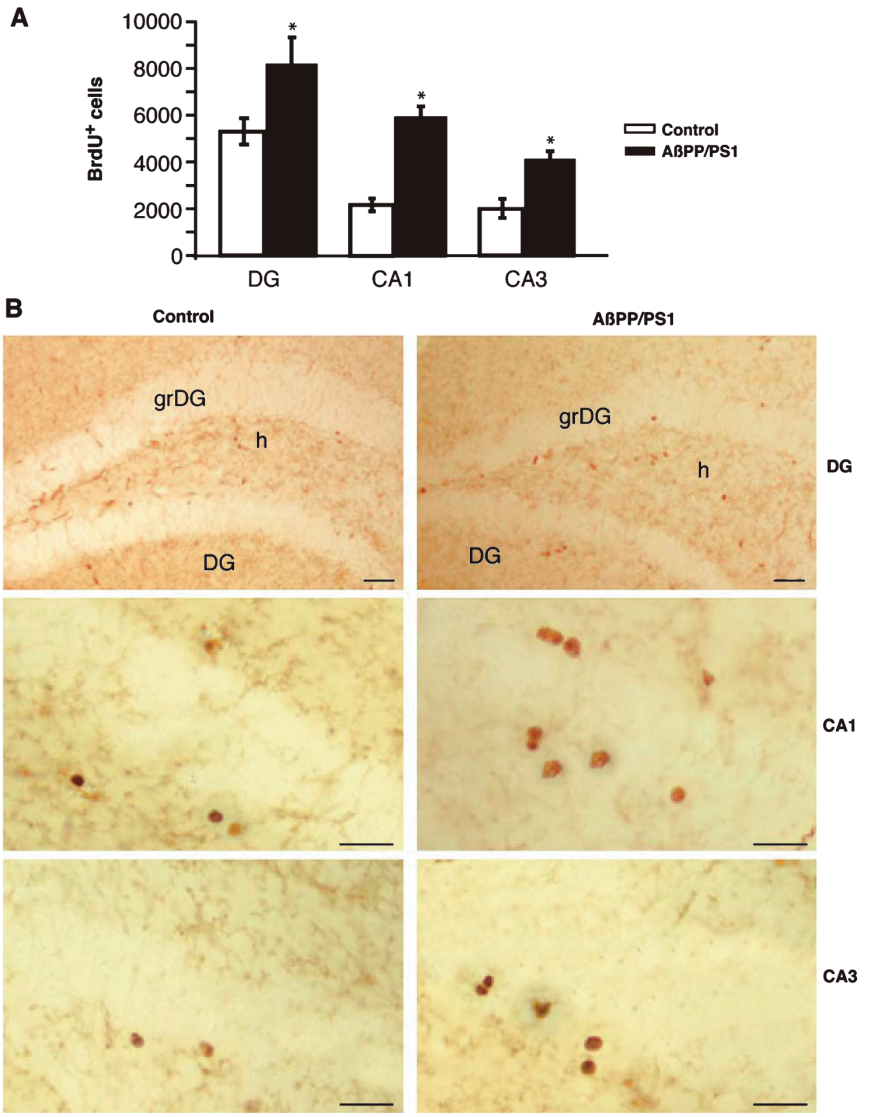

Fig. 6. Cell proliferation labelling by BrdU staining in the hippocampus of wild-type and AβPP/PS1 mice. A) Histograms show the number of $BrdU^+$ nuclei in control (white bars) and AβPP/PS1 (black bars). Data are expressed as mean ± standard error. $*p < 0.05$; (control mice $n = 8$; Tg mice $n = 7$). B) Photomicrographs, with different magnification, show $BrdU^+$ nuclei in the dentate gyrus (DG), CA1, and CA3 of control and AβPP/PS1. Scale bars = 20 μm. grDG: Granular layer of the dentate gyrus; h: hippocampus.

methods both to animals and humans [12, 30, 31]. Previous studies in mouse models of AD have mainly focused on the Aβ burden [32–34], volumetric changes [18, 35], or even MRS parameters, as predominant markers for AD [36, 37]. Here, we have implemented a different approach. We have applied a series of MRI/MRS methods to investigate the complete mouse brain and obtain a comprehensive database containing the most commonly used MRI/MRS biomarkers including T_2, MT, and ADC as well as regionalized cortical and subcortical MRS. We have then used a logistic regression algorithm to choose within all these, the

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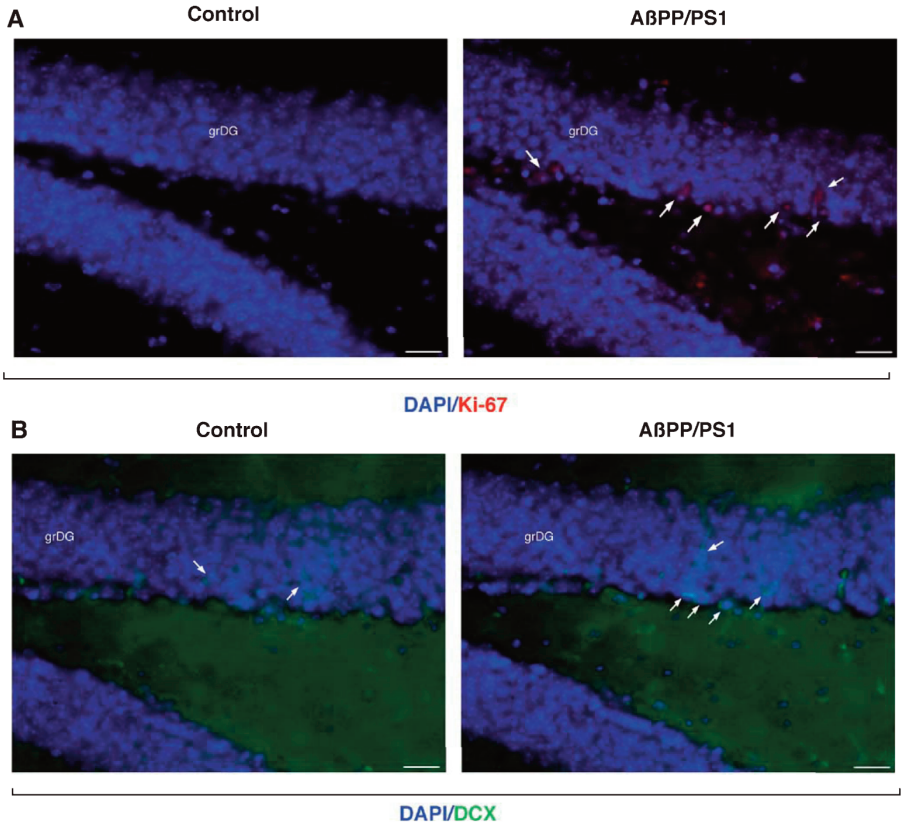

Fig. 7. Representative photomicrographs of Ki-67 and DCX in the DG of wild-type and AβPP/PS1 mice. Photomicrographs show (A) fluorescent Ki-67 (red) and (B) DCX (green) staining in the granule cell layer of the dentate gyrus in AβPP/PS1 mice. Nuclei were counterstained with DAPI (blue). Scale bars = 20 μm. grDG: Granular layer of the dentate gyrus.

biomarker or combination of biomarkers that provided the best discrimination between normal and double transgenic AβPP/PS1 mice.

In humans, diagnostic MRI examinations are normally targeted to the detection of medial temporal lobe (MTL) atrophy, as well as inflammatory glial infiltrations and neuronal degeneration. Inflammatory responses occur with an increase in cerebral water content normally resulting in astrocytic swelling. These effects may be visualized as increased T_2 and ADC values and decreased MT values in the corresponding images. We detect increased T_2 intensity values in the hippocampus and cortex of the AβPP/PS1 mice compatible with the signs of neuroinflammation and neurodegeneration previously described in this model of AD [23].

Ventricular enlargement is a MRI-based structural biomarker typically characterizing AD [30, 38]. Hemispheric atrophy rates, as measured by ventricular enlargement, correlate more strongly with changes on cognitive tests than MTL atrophy rates [39]. Moreover, ventricular enlargement and/or brain atrophy correlate well with declining performance in cognitive scales [40] and the rate of ventricular volume changes is highly correlated with an increase in Aβ-bearing plaques and NFTs [41]. In this study, we describe a significant ventricular enlargement in the AβPP/PS1 mice with the lateral ventricles being the most affected. Interestingly, ventricular engorgement has also been reported in a tau mouse model of AD [42]. It has been reported, however, that the pattern of lifetime ventricular expansion in mice differs from that found on humans, thus the physiological ventricular enlargement during normal aging must be taken into account in related experiments [43]. In our study, ventricular enlargement is accompanied by a discrete

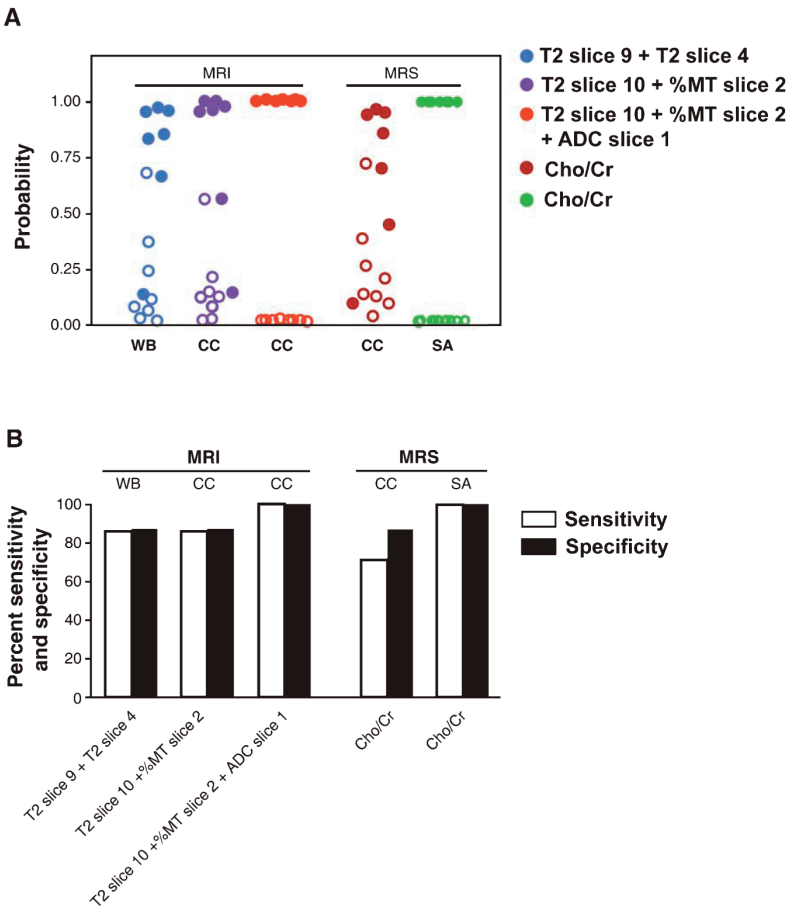

Fig. 8. Discriminatory potential of MRI and MRS parameters using logistic regression analysis. A) Scatter plots showing the individual posterior probability for being correctly classified as 0: wild-type mice or 1: AβPP/PS1 mice using different MRI and MRS combination of variables. The first parameter of each region corresponds with the one with the highest score, followed hierarchically by the next variables. Open symbols: control mice; filled symbols: transgenic animals. B) Sensitivity (white bars) and specificity (black bars) of control and AβPP/PS1 mice by using logistic regression analyses. WB: whole brain; CC: cerebral cortex; SA: subcortical area.

hippocampal volume decrease in the AβPP/PS1 mice. There are conflicting results in the literature regarding hippocampal changes in different mouse models of AD. While some authors reported decreased hippocampal volume in the PDAβPP or the AβPP/PS1 mice [44, 45], others reported enlarged hippocampus in the TASTPM mice [46]. Thus, genetic background or transgene expression, as well as other factors such as the age of animals or even the method of measurement, may play a role in the differences found. Taken together, it seems that ventricular enlargement, rather than hippocampal volume in transgenic mouse models of AD may have a better clinical translational potential for AD diagnosis.

MT imaging can provide pathophysiological information about the microstructure of the brain, reflecting the underlying histopathology changes. As previously reported for patients with AD [47–49], we detect a significant decrease in % MT in the cortex and hippocampus, as well as in the whole brain of the AβPP/PS1 mice. Localized alterations in the % MT have been associated with cognitive status in MCI and AD patients [50, 51]. The mechanisms underlying % MT reduction in the brain of AD patients are not completely understood, but they could involve changes in the relative ratio between free and macromolecule bound water molecules and their exchange occurring during inflammation. Additionally, it has been

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suggested that decreased % MT could reflect changes associated with neuronal loss, accumulation of plaques and NFTs, and gliosis [52].

By measuring ADC values, one can quantify alterations in apparent water diffusivity resulting from changes associated with brain injury and/or disease progression [53]. A trend towards higher ADC values is observed in whole brain as well as hippocampus or cortex of the AβPP/PS1, compared to control mice, but these trends are not statistically significant. Similar findings were reported in other AD mouse model that expresses a double mutant form of hAβPP 695 (K670 N/M671 L and V717F) [54].

In this work, we combine MRI analyses with additional MRS to unravel quantitatively metabolic and biochemical changes in the brain tissue *in vivo*. Reduction of NAA has been widely used as an indicator of brain pathology and of disease progression in AD patients [55–59]. Alterations in NAA/Cr have been also detected in the hippocampus of 6.5–9 month-old AβPP/PS1 mice [45]. In contrast, we do not observe significant changes in the NAA/Cr ratio in the cortical or subcortical regions of the AβPP/PS1 mice. Our results, however, are in agreement with previous reports in double transgenic mice (AβPP/PS1) that show decreased ratio of NAA/Cr, but only in mice > twelve months-old [36]. Age-dependent changes in brain NAA levels correlating with increasing amyloidosis have also been reported in the PS2AβPP mouse model of AD [37].

An increase in the ratio of Cho/Cr levels has been found in the cortex and subcortical areas of AβPP/PS1 mice compared with wild-type littermates. Changes in the concentration of Cho were not observed in previous mouse AD models [36]. This discrepancy in observed metabolites may arise from variations in the region of the brain sampled as well as from age or intrinsic differences among mouse models. In humans, there are conflicting reports about Cho levels in AD sufferers; whereas some authors found elevated Cho/Cr levels [60], others did not [61, 62]. Notably, wild-type and AβPP/PS1 mice were completely differentiated at twelve months of age, when Aβ deposits are widespread, by the ratio of Cho/Cr. This ratio could, therefore, provide a highly sensitive *in vivo* surrogate marker of disease status, at least in twelve month-old mice. Interestingly, a prospective study involving 509 elderly persons showed that persons with high Cho/Cr ratio had a higher risk to develop dementia within four years [63]. Moreover, it has been recently reported that high Cho/Cr ratios are associated with the preclinical pathologic processes in the AD cascade. Elevated Cho/Cr ratios correlated with decreased performance on domain-specific cognitive tests independent of Aβ load [64].

The higher Cho/Cr ratio in AβPP/PS1 mice correlates with enhanced neurogenesis as assessed by BrdU incorporation and the co-localization of the proliferation marker Ki-67 and the neuronal precursor marker DCX. Several mouse models of AD have been found to display altered adult neurogenesis, although there are conflicting results as to whether neurogenesis is decreased or increased [65, 66]. The increased proliferation of neuronal progenitors reported here in the twelve month-old AβPP/PS1 mice is in agreement with recent work indicating a role for leptin inducing similar cell proliferation in the hippocampus of the adult AβPP/PS1 mice [67], and with human postmortem brain study of AD patients that showed increased hippocampal neurogenesis in DG and CA1 [68]. An increase in the proliferation of neuronal progenitors may represent the compensatory response to brain damage; however, additional work is needed to elucidate if these progenitors are able to differentiate in mature functional neurons that successfully integrate into the adult mouse brain circuitry. Another possible explanation for the increased Cho levels in AD and in the AβPP/PS1 mice is that the Cho peak is the consequence of membrane phosphatidylcholine catabolism in order to provide free choline for the chronically deficient acetylcholine production in AD [69]. The fact that the increase of Cho/Cr levels in striatum of AβPP/PS1 mice is partially mitigated by treatment with the acetylcholine-esterase inhibitor donepezil [70] raises the possibility that Cho/Cr levels could be used as a biomarker of therapeutic efficacy in AD trials. This result is also in line with a study made in AD patients, showing decreased levels of Cho/Cr after four months of donepezil treatment [71].

Logistic regression analyses indicate that the most sensitive single measurement to discriminate control from AβPP/PS1 mice that are twelve months of age is the subcortical Cho/Cr ratio. The combination of MRI measurements in the cerebral cortex (T_2 slice 10 plus % MT slice 4 and ADC slice 1) also fully discriminates both groups of animals. Other measurements such as volumetric changes also provide useful information about disease status, although with lower sensitivity/specificity scores. Taken together, these observations highlight the clinical usefulness of MRS-based biomarkers.

An important limitation of the present study relies in that only twelve month-old mice were used. Therefore, the observed changes must be considered only

as potential biomarkers for disease status, supporting diagnosis, but their ability to monitor disease progression remains to be proven by longitudinal studies.

The AD model used in the present study was previously characterized by the amyloid burden, behavioral deficit, and neuronal degeneration and synaptic loss [20, 23, 24]. Together with the MRI/MRS methods described here, it now represents a well-characterized model for preclinical imaging and pathological studies of AD. Moreover, there is good agreement between changes observed in the brain of AβPP/PS1 mice here and those reported in humans. We believe then that the results reported herein may hopefully provide valid translational clues for AD evaluation as well as for the development and validation of novel therapeutic strategies.

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Discusión

5

1 Estudio de la actividad proliferativa y de la vulnerabilidad frente a la retirada de suero en células extraneurales de pacientes de EA

La primera parte del trabajo presentado en esta tesis se ha realizado en linfocitos obtenidos a partir de sangre periférica de pacientes de EA y donantes controles de edad similar inmortalizados con el virus de Epstein-Barr. Este virus, asociado con enfermedades como el linfoma de Burkitt, infecta selectivamente las células B e induce su proliferación, hecho que se transmite a la progenie, lo que permite inmortalizar los linfocitos y hacer que se dividan indefinidamente en cultivo (Zimber-Strobl y Strobl, 2001). Las líneas linfoblásticas resultantes expresan marcadores de activación y moléculas de adhesión similares a las células B activadas (Wang y cols., 1990), lo que ha permitido su uso como modelo experimental en diferentes estudios, entre ellos, diversos sobre la enfermedad de Alzheimer (Cecchi y Takahashi, 1999; Bojarski y cols., 2007; Chagnon y cols., 2008; Srimatkandada y cols., 2008; Zullo y cols., 2009; Zhang y cols., 2010). Es importante destacar que el proceso de transformación viral de las líneas linfoblásticas no altera la respuesta celular, ya que las principales características que hemos descrito en las líneas inmortalizadas se han encontrado también en linfocitos B no transformados (Bartolomé y cols., 2007; Muñoz y cols., 2008).

Empleando este modelo experimental hemos podido demostrar en nuestro laboratorio que los linfoblastos de EA muestran características neoplásicas, es decir, presentan una mayor actividad proliferativa cuando son estimulados por

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suero (Urcelay y cols., 2001; de las Cuevas y cols., 2003; Muñoz y cols., 2008) y son más resistentes a la muerte celular inducida por la retirada del mismo (Bartolomé y cols., 2007; Bartolomé y cols., 2010). Esta alteración de la respuesta celular de los linfoblastos se puede interpretar como una manifestación sistémica de la enfermedad y un reflejo de las alteraciones en el control del ciclo celular que se producen en neuronas postmitóticas y que están vinculadas a la muerte neuronal. Trabajos previos de nuestro laboratorio pusieron de manifiesto que el control del destino celular en presencia o ausencia de factores tróficos depende en último término de la regulación de los niveles de dos proteínas fundamentales para el control del ciclo celular como p27 y p21, respectivamente (Muñoz y cols., 2008; Bartolomé y cols., 2010). p27 y p21 son proteínas inhibidoras de las quinasas dependientes de ciclinas (Cdks) y juegan un papel fundamental en el control del tránsito G_1/S del ciclo (Currais y cols., 2009). Las rutas de señalización implicadas en la regulación de ambas proteínas en nuestras células dependen de la vía calcio/calmodulina (Ca^{2+}/CaM). En el caso de la respuesta proliferativa, en presencia de suero, Ca^{2+}/CaM interacciona con la vía PI3K/Akt, cuya sobreactivación se relaciona con un incremento en la degradación del inhibidor del ciclo p27. La disminución de los niveles de p27 se asocia, de esta forma, con un aumento en la proliferación (de las Cuevas y cols., 2003; Muñoz y cols., 2008). Por otro lado, en ausencia de suero, Ca^{2+}/CaM interacciona con la vía MAPK. Ca^{2+}/CaM, en un mecanismo mediado por CaMKII, regula negativamente los niveles de ERK1/2. La menor activación de ERK1/2 en las células de pacientes conduce en último término a un aumento en los niveles de p21 y a una mayor supervivencia frente a la retirada de suero (Bartolomé y cols., 2007; Bartolomé y cols., 2010). Un resumen de estos resultados se puede encontrar en la figura 24.

De acuerdo con estos resultados, el papel de Ca^{2+}/CaM es fundamental para la regulación de la respuesta de los linfoblastos a la presencia o ausencia de suero. Por ese motivo, el primer objetivo de esta tesis ha sido estudiar los mecanismos que regulan los niveles y la actividad de CaM en células de pacientes de EA e individuos control. En segundo lugar, se ha profundizado en el estudio de los mecanismos por los que la vía Ca^{2+}/CaM interacciona con las rutas PI3K/Akt y MAPK para controlar los niveles celulares de p27 y, especialmente, de p21, asociados con el aumento en la actividad proliferativa y la resistencia a la muerte celular tras la retirada de suero respectivamente.

1.1 Regulación del contenido de calmodulina en linfocitos inmortalizados de pacientes de alzhéimer.

La calmodulina es la principal proteína de unión a calcio de la célula. Es una proteína pe-

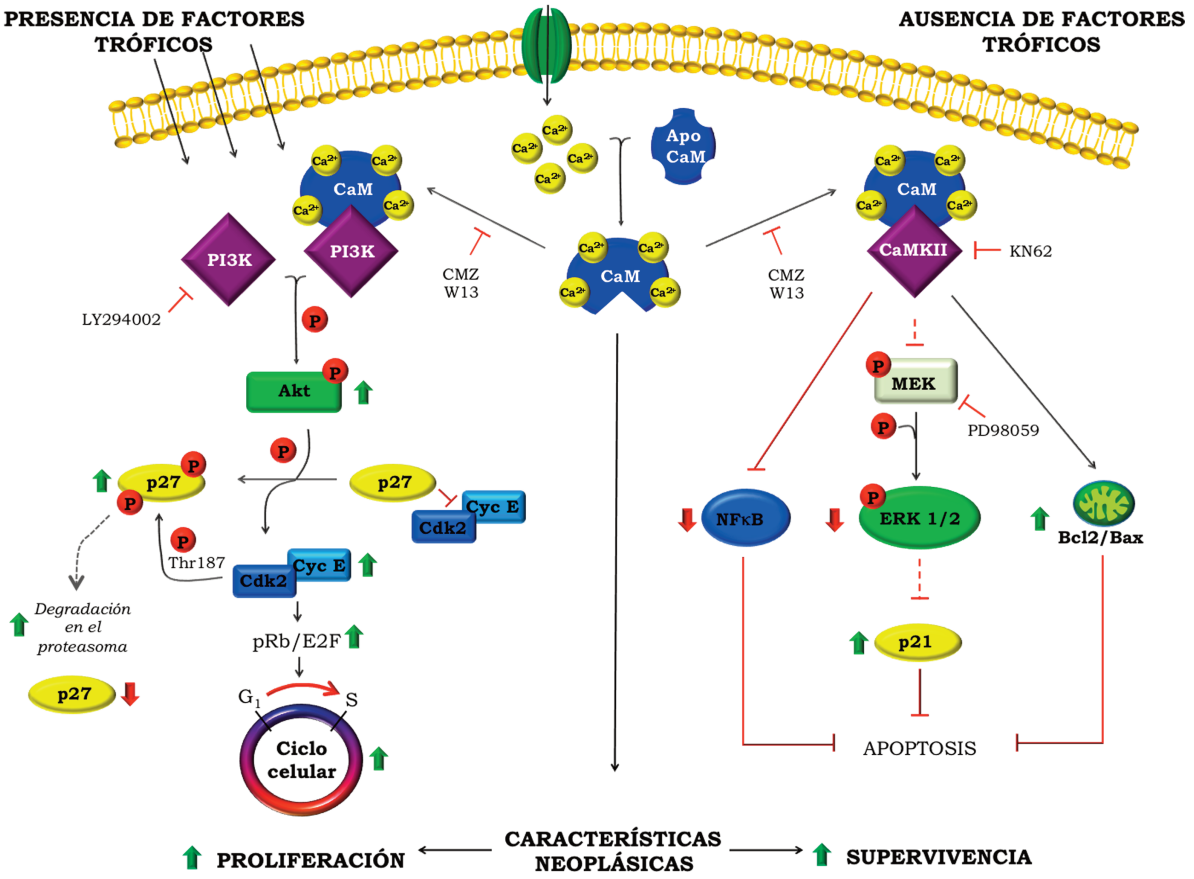

Figura 24. Esquema de las rutas implicadas en la mayor proliferación y supervivencia de los linfoblastos de EA. En las células de EA, la presencia de suero promueve la sobreactivación dependiente de Ca^{2+}/CaM de la vía PI3K/Akt que conduce a una mayor degradación de p27, a una mayor actividad de ciclina E/Cdk2/pRb/E2F y una mayor entrada en ciclo. En ausencia de suero, la ruta Ca^{2+}/CaM, a través de CaMKII, disminuye la activación de NF-κB y ERK1/2 en comparación con controles, e incrementa el contenido celular de p21, que parece proteger a los linfoblastos de EA de la apoptosis inducida por la ausencia de factores tróficos.

queña, de 17 kDa, compuesta por 148 aminoácidos y formada por una única cadena polipeptídica que presenta un dominio globular N-terminal y un dominio globular C-terminal formados cada uno por dos motivos *mano EF*, una estructura formada por dos alfa hélices que permite la unión de un ión Ca^{2+} (Yamniuk y cols., 2007). Un incremento en los niveles de calcio intracelular produce la unión secuencial de hasta cuatro iones de Ca^{2+} en la proteína, lo que desencadena una serie de cambios conformacionales, que conducen a la exhibición de residuos clave en una conformación conocida como "forma abierta" y que le permiten unir y activar sus múltiples proteínas diana, conocidas como CaMBP (*CaM-Binding Proteins).* Entre otras CaMBP destacan la fosfatasa calcineurina o diver-

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sas quinasas, entre ellas la familia de quinasas dependientes de Ca^{2+}/CaM, como CaMKII (Yamniuk y Vogel, 2004). CaM interviene de esta manera en numerosos procesos fisiológicos, como la progresión del ciclo celular. Se ha descrito que existe una relación entre los niveles celulares de CaM y la capacidad proliferativa de la célula, de manera que un mayor contenido en CaM se asocia con mayor proliferación (Rasmussen y Means, 1987; Kahl y Means, 2003).

Nuestros resultados indican, en primer lugar, que los linfocitos de pacientes de EA presentan un mayor contenido en CaM que las células control y que este hecho no se debe a una mayor expresión de los genes que la codifican, sino a una disminución en su tasa de degradación. En el primer caso, el estudio por PCR cuantitativa de los niveles de ARN mensajero de los tres genes que codifican CaM en mamíferos (*CALM1, CALM2 y CALM3* (Berchtold y cols., 1993)) señala que el incremento en los niveles celulares de CaM en linfoblastos de pacientes no se debe a un aumento en su expresión, ya que los niveles de ARN mensajero son similares a los de las células control. Sin embargo, el tratamiento de las células con ciclohexamida para inhibir la síntesis de proteínas *de novo* desvela que la tasa de degradación de CaM en células de pacientes es mucho menor que en controles. Estimamos que la vida media de CaM en células de EA es de aproximadamente 22 horas, tres veces mayor que en individuos control. Este resultado es similar a otros descritos para la vida media de la proteína en cerebro de rata (Ferrington y cols., 1998). Además, es interesante señalar que tiene una vida media mucho menor que otras proteínas relacionadas con la homeostasis del calcio, como la Ca^{2+}-ATPasa de la membrana plasmática, para la que se ha descrito una vida media de 12 días en ese mismo tejido (Ferrington y cols., 1998). En cualquier caso, los valores son similares a los de la mayoría de proteínas celulares.

Hemos descrito que la degradación de calmodulina en linfocitos inmortalizados se produce en el proteasoma. En efecto, se produce una acumulación de la proteína tanto en células control como en linfoblastos de pacientes, cuando inhibimos selectivamente el protesoma con lactacistina. Sin embargo, la inhibición de otros mecanismos de degradación de proteínas, como las caspasas o la autofagia, no afecta a los niveles de CaM. Los ensayos de coinmunoprecipitación de CaM y ubiquitina muestran que la proteína se ubiquitina en estas líneas celulares. Aparentemente, se produce una ligera disminución de la poliubiquitinación de CaM en las células de pacientes de EA que quizá pueda contribuir a disminuir la velocidad de degradación de la molécula. Sin embargo, algunos autores han descrito que la proteína "envejecida" u oxidada se puede degradar en los proteasomas 20S y 26S sin necesidad de ubiquitinarse (Tarcsa y cols., 2000; Balog y cols., 2009), y que la ubiquitinación no aumenta su tasa de degradación (Bena-

roudj y cols., 2001). Es probable que ambos mecanismos, tanto los dependientes de ubiquitina como los no dependientes, colaboren en la degradación de la proteína. Serían necesarios más estudios para evaluar la contribución potencial de ambos en el control de los niveles de CaM.

Aunque se han descrito alteraciones en la funcionalidad del proteasoma en el cerebro de pacientes de EA (Keller y cols., 2000; Keck y cols., 2003), trabajos previos de nuestro y de otros laboratorios no han mostrado deficiencias en la actividad del mismo en linfocitos de afectados de EA (Blandini y cols., 2006; Muñoz y cols., 2008; Ullrich y cols., 2010). En nuestro laboratorio, además, se ha descrito un aumento en la tasa de degradación en el proteasoma de otro inhibidor del ciclo como p27 (Muñoz y cols., 2008). Por otra parte, nuestros resultados indican que la tasa de degradación de β-actina es similar en las células de controles y de pacientes. Por tanto, la disminución de la velocidad de degradación en linfoblastos de EA no se puede atribuir a alteraciones inespecíficas de los sistemas de degradación de proteínas.

Dos características fisiopatológicas importantes de la enfermedad, como las alteraciones en los niveles de ROS o en la homeostasis del calcio (Zhu y cols., 2007b; Yu y cols., 2009), también se reflejan en los linfoblastos de los pacientes. Por un lado, nuestros resultados indican que las células de pacientes de EA presentan mayores niveles de ROS que las células control. Por otro lado, a pesar de presentar mayores niveles de CaM, los linfoblastos de EA muestran una mayor concentración intracelular de calcio. Este hecho estaría de acuerdo con resultados previos que muestran una menor capacidad de los linfoblastos de EA para tamponar los incrementos citosólicos de Ca^{2+} (Ibarreta y cols., 1997). También se ha descrito una pérdida de la capacidad tamponadora de Ca^{2+} en cerebros de pacientes, que se relaciona con la vulnerabilidad neuronal selectiva y la neurodegeneración (Palop y cols., 2003). De modo que los cambios en los flujos iónicos de Ca^{2+} y en la actividad de las proteínas tamponadoras de estos iones serían responsables del incremento en la concentración de Ca^{2+} citosólico característica de los pacientes de EA (Berridge, 2010).

Tanto los niveles de ROS como los de calcio intracelular regulan en estas líneas linfoblásticas la tasa de degradación de CaM. El tratamiento de las células de EA con el quelante BAPTA para tamponar el calcio intracelular aumenta la tasa de degradación de CaM hasta niveles semejantes a los de las células control, mientras que el tratamiento de los controles con el ionóforo ionomicina, incrementa los niveles de calcio intracelular al mismo tiempo que aumenta la vida media de CaM. La reducción de la tasa de degradación de CaM podría interpretarse como una respuesta de las células al aumento de la concentración de Ca^{2+} citosólico. En este sentido, se ha descrito que la molécula nativa de CaM, apo-CaM, se degrada

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más rápidamente que la unida a Ca^{2+} (Benaroudj y cols., 2001). Experimentos *in vitro* muestran también que el calcio regula de manera opuesta los procesos de ubiquitinación y degradación de CaM. Mientras que por un lado la adición de calcio supone una mayor ubiquitinación de la molécula, por otro lado produce una disminución en su velocidad de degradación (Tarcsa y cols., 2000). Es posible que la molécula de CaM ubiquitinada pueda retener suficiente capacidad de unir Ca^{2+} para mantener una estructura demasiado rígida que impida su plegamiento y ser dirigida al proteasoma (Tarcsa y cols., 2000). Por otra parte, el tratamiento de los linfoblastos de pacientes con dos agentes antioxidantes como trolox y glutation reducido (GSH), disminuye los niveles de ROS y acelera la degradación de la proteína. Es sabido el efecto que tienen los niveles altos de ROS en los flujos de Ca^{2+} (Yu y cols., 2009a; Peng y Jou, 2010). Por tanto, cabe pensar que este efecto de los niveles de ROS alterando la degradación de CaM juegue un papel adicional en la influencia que éstos ejercen en la homeostasis del calcio.

La ruta de señalización dependiente de Ca^{2+}/CaM parece jugar un papel importante en la patología de la EA. Se ha descrito que numerosas proteínas implicadas en la enfermedad (muchas relacionadas con el procesamiento de APP y tau) presentan dominios de unión a CaM, con lo que pueden ser potenciales CaMBPs que actúen y se regulen dependiendo de la señalización de esta vía (O'Day y Myre, 2004). A pesar de ello, los mecanismos que regulan los niveles de CaM no han sido estudiados en profundidad en la EA. El aumento en los niveles de CaM que encontramos en los linfoblastos de pacientes contrasta con otros trabajos en los que se observa una reducción de la proteína en la corteza cerebral de material de autopsia de afectados de EA (Solomon y cols., 2001). Esta discrepancia quizá pueda atribuirse a que algunos anticuerpos contra CaM son capaces de reconocer sólo la proteína en determinados estados conformacionales.

1.2 Interacción de la vía Ca^{2+}/CaM con la ruta PI3K/Akt en el control de la actividad proliferativa de los linfoblastos de EA

Como se ha mencionado con anterioridad, la unión de hasta cuatro iones de Ca^{2+} a la molécula de CaM induce una serie de cambios conformacionales que aumentan la exposición de residuos hidrofóbicos que sirven de sitios de unión para las distintas dianas proteicas de CaM. También se ha descrito que tanto la saturación de Ca^{2+} como la localización de los iones Ca^{2+} unidos a CaM influyen en la capacidad de la proteína de unirse a sus dianas (Schumacher y cols., 2001).

Nuestros resultados han permitido demostrar que el aumento de los niveles de CaM en linfoblastos de EA es el responsable de la mayor actividad de dos proteínas clave en los procesos

que regulan la proliferación/supervivencia/muerte celular, como PI3K y CaMKII.

La primera de ellas, PI3K, media la señalización de diferentes procesos celulares y consta de dos subunidades, la reguladora o p85 y la catalítica o p110. La unión de la subunidad p85 a distintas proteínas señal, libera p110 y permite activar la subunidad catalítica (Engelman y cols., 2006) que cataliza la reacción que da como productos fosfatidil inositol 3-4 bisfosfato (PIP_2) e inositol 3,4,5 trifosfato (PIP_3). PIP_2 y PIP_3 se unen a un subgrupo de proteínas entre las que se encuentra la serina-treonina quinasa Akt. Akt contiene dominios de pleckstrina, que permiten su unión a los fosfolípidos y le sirven de anclaje, facilitando su translocación a la membrana plasmática donde es fosforilada y activada (Song y cols., 2005).

Nuestros resultados demuestran que, en linfoblastos de pacientes de EA, la sobreactivación de la vía PI3K/Akt está asociada al aumento de la actividad proliferativa a través de un mecanismo que en último término induce la degradación del inhibidor de Cdks p27 y que es sensible a los antagonistas de CaM, en consonancia con el papel que juega CaM en la regulación del ciclo celular (Kahl y Means, 2003). Los resultados presentados en esta tesis muestran que CaM es capaz de unirse a la subunidad reguladora p85 de la PI3K, que esta unión se produce en mayor medida en los linfoblastos de EA que en los controles y que, además, es una interacción calcio-dependiente. Estos resultados están de acuerdo con estudios previos que demostraban que CaM interaccionaba con los dominios SH_2 de la subunidad p85, aumentando su actividad *in vitro* y en cultivos celulares (Joyal y cols., 1997; Perez-Garcia y cols., 2004). Otros autores han descrito la capacidad de unión directa de CaM con Akt a través de los residuos pleckstrina (Dong y cols., 2007).

La sobreactivación de la vía PI3K/Akt también se ha podido demostrar en cerebros de EA, asociada con un aumento en la fosforilación de GSK3β y mTOR y una disminución en los niveles de p27 (Griffin y cols., 2005). Se ha considerado que la sobreactivación de la vía PI3K/Akt, normalmente asociada al control de la supervivencia celular en cerebros de pacientes de EA, pudiera ser una respuesta de las neuronas para compensar los daños producidos por la enfermedad. Recientemente se ha descrito que la activación de Akt puede ser clave para las neuronas en el proceso de abortosis, por el cual no completan la cascada apoptótica (Liu y cols., 2012). Sin embargo, el incremento en la activación de esta vía en neuronas postmitóticas puede también inducir la entrada en ciclo y la muerte celular como consecuencia de sus efectos sobre proteínas reguladoras del ciclo celular.

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1.3 Interacción de la vía Ca^{2+}/CaM con la ruta MAPK para la regulación del contenido celular de p21 y el control de la vulnerabilidad frente a la retirada de suero.

Nuestros resultados también muestran que el aumento de los niveles de CaM en los linfoblastos de EA se acompaña de una mayor actividad de CaMKII. CaMKII es una serina/treonina quinasa regulada por el complejo Ca^{2+}/CaM que interviene en diversas rutas de señalización. En nuestro laboratorio se había descrito previamente que esta proteína juega un papel muy importante en el control de la respuesta celular a la apoptosis inducida por la retirada de suero en linfocitos inmortalizados de pacientes de EA (Bartolomé y cols., 2007; Bartolomé y cols., 2010). Nuestros resultados muestran que la ruta Ca^{2+}/CaM/CaMKII interacciona con la vía MAPK para regular los niveles de p21 y la vulnerabilidad celular frente a la privación de suero. La retirada de suero induce en los linfoblastos la activación sostenida de ERK1/2. Sin embargo, CaMKII regula negativamente este proceso, de manera que la activación de ERK1/2 es significativamente menor en los linfoblastos de EA 72 horas después de la retirada de suero. Los resultados presentados en esta tesis muestran que la regulación negativa de ERK1/2 por CaMKII aumenta los niveles de p21 y la resistencia frente a la muerte celular de los linfoblastos de EA al favorecer la actividad del factor de transcripción FOXO3a *(Forkhead Box O 3a)*. Estas afirmaciones se basan en las siguientes observaciones: 1) existe una relación inversa entre la actividad de CaMKII y ERK1/2, de manera que la actividad de CaMKII es mayor en las células de pacientes de EA y se acompaña de una menor fosforilación de ERK1/2, de una mayor localización nuclear de FOXO3a y de una mayor expresión de p21. 2) La inhibición de CaMKII en los linfoblastos de pacientes restaura los niveles de fosforilación de ERK1/2 y disminuye los niveles nucleares de FOXO3a y la expresión de p21, sensibilizando a las células frente a los efectos apoptóticos que produce la retirada de suero. 3) El inhibidor de ERK1/2 PD98059 previene la muerte de los linfoblastos control en ausencia de suero al mismo tiempo que aumenta el contenido de FOXO3a en el núcleo e induce la expresión de p21.

Como señalan nuestros resultados, el mayor contenido de p21 en las células de pacientes tras la retirada de suero se relaciona con el aumento en los niveles de ARN mensajero en un proceso regulado negativamente por ERK1/2. De hecho, aunque los mecanismos post-transcripcionales y post-traduccionales son importantes en la regulación de p21, la regulación transcripcional es el principal punto de control de la expresión de la proteína y se puede producir por diversos mecanismos dependientes e independientes del supresor tumoral p53 (Jung y cols., 2010). Nuestros resultados muestran que, a pesar del mayor contenido en p21, los linfoblastos de pacientes presentan niveles semejantes de p53 total o

fosforilada en serina 15 que los controles y que el tratamiento con el inhibidor de p53, pifithrin α, tiene escaso efecto en los niveles de p21 y no afecta a la supervivencia de las células, descartando así la implicación de p53 en la regulación de la expresión de p21 en linfocitos inmortalizados. Entre los mecanismos independientes de p53, se había descrito que el factor de transcripción FOXO3a podía mediar la activación transcripcional de p21 en algunos tipos de células (Hauck y cols., 2007) y que, además, este factor de transcripción se regula negativamente tras la activación de ERK1/2 (Yang y cols., 2008b). FOXO3a es un miembro de la clase O de la familia de factores de transcripción *forkhead*, que se expresa en diferentes tejidos y media los efectos de los factores de crecimiento en muchos procesos celulares como la progresión del ciclo, el metabolismo de glucosa y la apoptosis (Kops y cols., 2002; Accili y Arden, 2004; Greer y Brunet, 2008). Su actividad transcripcional depende mayoritariamente, aunque no exclusivamente, de su localización intracelular (Vogt y cols., 2005). En ausencia de factores tróficos, FOXO3a se localiza preferentemente en el núcleo celular, induciendo la transcripción de una gran variedad de genes (Modur y cols., 2002). Nuestros resultados indican que el contenido celular de FOXO3a está aumentado en linfoblastos de EA privados de suero. En estas condiciones, FOXO3a se acumula en el núcleo y aumenta la transcripción de p21, favoreciendo la resistencia a la muerte celular inducida por la retirada de suero. Aparentemente, los niveles de FOXO3a son regulados por la vía CaM/CaMKII/ERK1/2 a través de la fosforilación de la molécula y su posterior degradación en el citosol en un mecanismo mediado por MDM2 (*Murine-Double Minute 2)*.

Numerosas evidencias experimentales apoyan la idea de que las llamadas quinasas oncogénicas, como PI3K/Akt, IKK y ERK1/2 están involucradas en el control de la localización nuclear y la actividad de FOXO3a a través de la fosforilación de residuos específicos, lo que favorece la salida de la proteína del núcleo al citosol y su degradación (Plas y Thompson, 2003; Hu y cols., 2004; Yang y cols., 2008b). En los linfoblastos de pacientes de EA, sin embargo, la inhibición de la vía PI3K/Akt no tiene consecuencias en la transcripción de p21 ni en la supervivencia celular frente a la retirada de suero. Nuestros resultados indican que es la inhibición de ERK1/2 la que regula la localización nuclear de la proteína al parecer previniendo la fosforilación del factor e impidiendo su salida al citoplasma y su degradación. Al no poder encontrar un anticuerpo adecuado contra FOXO3a fosforilado en los residuos que fosforila ERK1/2 (Yang y cols., 2008b), estudiamos el efecto de la modulación de los niveles de ERK1/2 en el contenido nuclear de FOXO3a. Encontramos que la inhibición de ERK con PD98059 favorece la acumulación de FOXO3a en el núcleo, mientras que la estimulación de ERK1/2, al inhibir CaMKII con CMZ y KN62, dis-

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minuye el contenido nuclear de FOXO3a. Se ha descrito, además, que ERK1/2 regula la proteína MDM2, que, entre otras funciones, puede actuar de ubiquitina ligasa E3, marcando diversas proteínas para su posterior degradación (Ries y cols., 2000; Phelps y cols., 2005). Entre otras, se ha descrito que puede mediar la degradación de FOXO3a en el sistema ubiquitina-proteasoma (Yang y cols., 2008b). Nuestros resultados indican que las células de los individuos control presentan una mayor activación de ERK1/2 unida a un mayor contenido en MDM2, y que en los linfoblastos de pacientes, donde ERK1/2 está menos activada, o tras la inhibición de la misma en los controles con PD98059, los niveles de MDM2 son menores. Estos datos apoyan la teoría de que los niveles de FOXO3a se controlan en estas células modulando la fosforilación de la molécula por la ruta CaM/CaMKII/ERK1/2, y su posterior degradación, como consecuencia de alterar la salida de la proteína del núcleo al citosol y los niveles de MDM2.

Además de observar un aumento en la transcripción de p21 en los linfoblastos de pacientes de EA, también hemos encontrado un incremento en los niveles citosólicos de la proteína tras la retirada de suero. p21 puede presentar distintas funciones dependiendo de su localización subcelular. Además de su función clásica como proteína inhibidora de Cdks en el núcleo, se ha descrito que, en el citoplasma, p21 protege a la célula frente a la apoptosis (Blagosklonny, 2002; Coqueret, 2003).

Por otro lado, no hemos observado cambios en la tasa de degradación de p21 tras la retirada de suero en nuestras células. Estos resultados contrastan con otros previos de nuestro laboratorio, en los que se había observado una mayor degradación de p21 en condiciones proliferativas en presencia de suero (Sala y cols., 2008). Por lo tanto, parece que la adición o la retirada de suero producen efectos opuestos en los niveles celulares de p21 regulando la degradación o la transcripción y la localización respectivamente.

La relación entre los niveles elevados de p21 y la mayor resistencia frente a la retirada de suero en los linfoblastos de EA está de acuerdo con otros resultados en los que se ha descrito que un incremento en la expresión de p21 bloquea la muerte inducida por estrés oxidativo en células de mieloma humano U266 (Kim y cols., 2001) o que la inducción de la expresión de p21 (Gareau y cols., 2011) o de su localización citoplasmática (Koster y cols., 2010; Xia y cols., 2011) se relaciona con la resistencia a los quimioterápicos en algunos tipos de cáncer. Por lo tanto, se puede asumir que el aumento en los niveles de p21 proporciona a los linfoblastos de EA una ventaja para la supervivencia, de forma similar a lo descrito para las células tumorales (Weiss, 2003).

p21 puede proteger a las células de la apoptosis a través de distintos mecanismos. Puede interaccionar e inhibir distintas caspasas (Suzuki y cols., 2000; Roninson, 2002), unirse a

E2F1 y MYC e impedir la transcripción de proteínas pro-apoptóticas (Abbas y Dutta, 2009) y también inducir la expresión de proteínas anti-apoptóticas (Dotto, 2000). También es interesante la observación de que el aumento del contenido de p21 en fibroblastos de pacientes de EA se ha asociado con la inhibición de la apoptosis inducida por estrés oxidativo (Naderi y cols., 2006). En este sentido, es importante destacar que existen diferencias en la susceptibilidad de las células periféricas de los pacientes de EA y controles a la muerte celular inducida por diversos estímulos (Eckert y cols., 2001a; Morocz y cols., 2002; Uberti y cols., 2002; Naderi y cols., 2006), aunque existen discrepancias acerca de si las células de los pacientes son más resistentes o más vulnerables a los distintos inductores de muerte celular. Las diferencias pueden deberse a los distintos tipos celulares o inductores de muerte empleados.

Mientras que en respuesta al suero la mayor proliferación de los linfoblastos de pacientes de EA se relaciona con la sobreactivación de la vía PI3K/Akt, el control de la supervivencia/muerte celular se relaciona con la actividad de CaMKII, ya que la inhibición selectiva de esta enzima aumenta la vulnerabilidad de las células de EA frente a la muerte celular inducida por la retirada de suero hasta los valores observados en las células control. Los efectos la inhibición directa de CaMKII con KN62 se acompañan de la activación de ERK1/2, y de un menor contenido nuclear de FOXO3a y de p21. La inactivación de CaMKII no tiene consecuencias en las células control, lo que sugiere que existe un umbral de CaM por encima del cual las células se protegen contra la apoptosis inducida por la retirada de suero. Estos resultados están en consonancia con publicaciones previas que indicaban que los antagonistas de CaM bloqueaban la apoptosis en células de tumor de mama sin afectar a las células epiteliales normales de la glándula mamaria (Deb y cols., 2004).

Los mecanismos por los cuales la activación de CaMKII disminuye la actividad de ERK1/2 no se conocen con precisión. La cascada de las MAPK regula un gran número de procesos celulares que en general se asocian a la activación de receptores tirosina-quinasa y receptores acoplados a proteínas G. A grandes rasgos, la activación de la ruta de las MAPK comienza con la activación de Ras, una pequeña GTPasa unida a la membrana, que puede alternar entre la forma inactiva, unida a GDP y la forma activa unida a GTP. Una vez activa, recluta la quinasa Raf a la membrana, y ésta inicia una cascada de fosforilaciones, activando las quinasas MEK, que posteriormente fosforilan y activan ERK1/2. Se han descrito distintos mecanismos por los cuales CaMKII puede regular estas proteínas. Por un lado, se ha observado que en algunos tipos celulares, CaMKII fosforila la proteína Raf-1, favoreciendo la activación de ERK1/2 (Illario y cols., 2003; Salzano y cols., 2012). Por otro lado también se ha descrito que CaM puede unirse a K-

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Ras e inhibir la ruta Ras/Raf/MEK/ERK1/2 en fibroblastos (Villalonga y cols., 2001). CaMKII también puede activar *in vitro* la proteína SynGAP *(Synaptic Ras GTPase activating protein 1)* (Oh y cols., 2004) una proteína sináptica que activa la actividad GTPasa de Ras catalizando de esta forma su inactivación y la inhibición de ERK1/2 (Kim y cols., 1998). También en neuronas se ha propuesto un papel regulador de CaM de la actividad de K-Ras, mediante el cual CaM se puede unir a K-Ras, inhibiendo su fosforilación por distintas quinasas. La inhibición de CaM en estos casos aumenta la activación de K-Ras endógeno (Alvarez-Moya y cols., 2010). Aparentemente, CaM se une al extremo carboxilo terminal que ancla K-Ras a la membrana en un mecanismo en el que es muy importante la farnesilación de K-Ras (Wu y cols., 2011). Son necesarios más estudios para averiguar si estos mecanismos son operativos en linfocitos humanos y si están alterados en la EA.

Recientemente se ha descrito la existencia de un pool de genes, importante en la respuesta adaptativa y en la neuroprotección, cuya regulación es calcio-dependiente (Zhang y cols., 2009). Este sistema es extraordinariamente sensible a cambios en la señalización nuclear de calcio, por lo que se debe también considerar la posibilidad de que además del efecto indirecto de CaM/CaMKII en los niveles de FOXO3a y p21 a través de ERK1/2, estos genes pertenezcan al nombrado pool.

Los resultados obtenidos en células extraneurales de pacientes de EA no son directamente extrapolables a lo que sucede en el cerebro. Sin embargo, es interesante destacar que alteraciones similares a las descritas en linfoblastos se han detectado en cerebros de pacientes de EA. Se han descrito cambios en la activación de CaMKII relacionados con una mayor fosforilación de tau y una mayor formación de ovillos neurofibrilares (McKee y cols., 1990). CaMKII también se ha relacionado con el aprendizaje y la memoria y se ha descrito una disfunción en la distribución celular de p-CaMKII en las sinapsis que puede estar implicada en la disfunción cognitiva en los pacientes de EA (Reese y cols., 2011). De manera similar, se han encontrado alteraciones en la actividad de ERK1/2, generalmente asociadas a una mayor activación, en neuronas afectadas por EA (Zhu y cols., 2002). La ruta ERK1/2 parece estar implicada en la patogénesis de EA, por diversos mecanismos. Se ha descrito que puede mediar la neurotoxicidad de Aβ (Chong y cols., 2006), de tau (Pei y cols., 2002) y alterar la plasticidad neuronal en el hipocampo, contribuyendo a la pérdida de memoria (Derkinderen y cols., 1999). También se ha descrito un aumento en la actividad de FOXO3a en el cerebro del modelo transgénico de EA Tg2576, comparado con la actividad de este factor en ratones control, que se relaciona con un aumento en los niveles de Aβ40 y Aβ42 (Qin y cols., 2008). De la misma manera, se han encontrado elevados niveles de p21

en la corteza cerebral de pacientes de EA (Engidawork y cols., 2001). Nuestros resultados sugieren que la alteración del eje Ca^{2+}/CaM/CaMKII/ERK1/2 no es exclusiva de las neuronas, sino que también ocurre a nivel sistémico.

En resumen, los datos aquí presentados muestran que, en ausencia de suero, la supervivencia de los linfoblastos de EA depende de la disminución de la actividad de ERK1/2 inducida por CaMKII. El resultado es una acumulación de FOXO3a en el núcleo, consecuencia de la inhibición de la fosforilación y degradación del factor de transcripción y la posterior inducción transcripcional de p21 como se observa en la figura 25. De manera que la relación inversa entre CaMKII y ERK1/2 y entre ERK1/2 y FOXO3a controla el destino celular de los linfoblastos de pacientes de EA en función de la disponibilidad de factores tróficos.

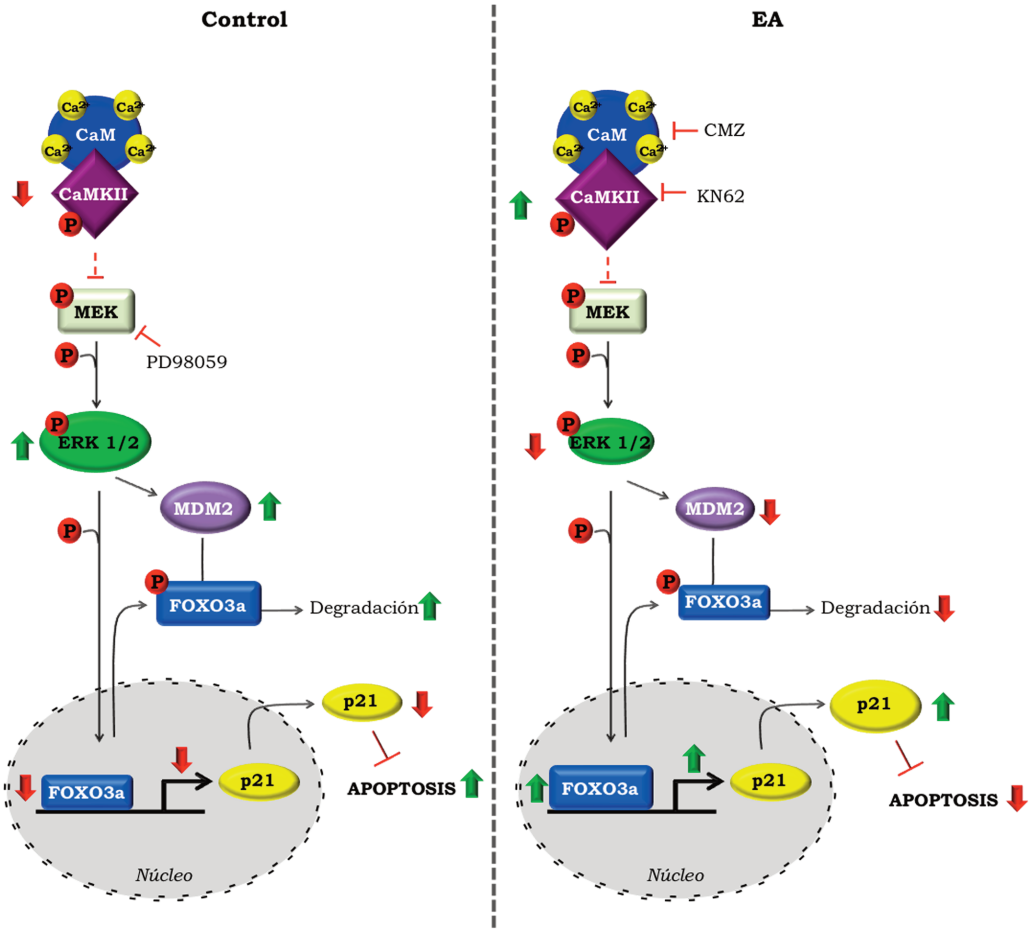

Figura 25. Esquema del papel de CaMKII/ERK1/2/FOXO3a en la regulación de los niveles de p21 y la supervivencia en linfocitos inmortalizados de pacientes de EA. En los linfoblastos de pacientes control, la retirada de suero favorece la activación de ERK1/2, que presumiblemente induce la degradación de FOXO3a, mediando su fosforilación y posterior salida al citoplasma y aumentando los niveles de MDM2. Estos procesos se revierten con el inhibidor de MEK PD98059. En linfoblastos de pacientes de EA, la retirada de suero regula negativamente ERK1/2 a través de una mayor activación de CaMKII. Este hecho previene la fosforilación y degradación de FOXO3a , favoreciendo su presencia en el núcleo, y aumentando de esta manera la transcripción de p21. p21 se localiza mayoritariamente en el citoplasma de estas células y las protege frente a la apoptosis. Estos efectos se revierten inhibiendo CaMKII con CMZ y KN62.

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La segunda parte de esta tesis se ha desarrollado empleando un modelo transgénico de la enfermedad, el ratón APP/PS1. A pesar de que ninguno de los modelos transgénicos existentes en la actualidad reproduce todas las características neuropatológicas que presentan los pacientes de EA, como la muerte neuronal o el desarrollo de ovillos neurofibrilares, el empleo de estos animales está siendo muy útil para el estudio muchos aspectos relacionados con la enfermedad y para valorar posibles tratamientos terapéuticos. En nuestro caso, el empleo de estos ratones nos ha permitido realizar un estudio comparativo de la posible alteración simultánea en el control del ciclo celular en el SNC y en células periféricas.

El modelo que hemos empleado es el ratón APP/PS1, un modelo doble transgénico, resultado del cruce entre los ratones Tg2576, que sobreexpresan la APP humana con la doble mutación sueca (K670N, M671L) y el ratón PS1, que porta la mutación (M146L) en presenilina 1. El ratón APP/PS1 resultante se emplea habitualmente como modelo transgénico de la enfermedad. En nuestro caso, hemos usado ratones de 12 meses de edad, que garantizan el depósito de amiloide y los signos de muerte celular por apoptosis en la corteza y el hipocampo (Yang y cols., 2008a; Antequera y cols., 2009; Spuch y cols., 2010).

En primer lugar, empleamos la tecnología de arrays de PCR (RT^{2}Profiler^{TM}-PCR Array) para

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cuantificar y comparar la expresión de 84 genes relacionados con el control del ciclo celular en el cerebro y células mononucleares de sangre periférica (CMSP) de ratones APP/PS1 respecto a ratones control. Por el momento, hasta donde nosotros conocemos, es la primera demostración de la existencia de alteraciones simultáneas en el control del ciclo celular en cerebro y células periféricas de un modelo murino de EA. Nuestros resultados indican que el genotipo APP/PS1 induce alteraciones en un amplio número de genes relacionados con el ciclo celular en ambos tejidos. El número de genes cuya expresión se ve alterada es mayor en CMSPs que en el cerebro (51% frente a 21%) y solamente 13 de ellos son comunes a los dos tejidos. Además, entre los genes comunes, algunos de ellos cambian en direcciones opuestas, lo que sugiere que su expresión puede estar regulada de forma dependiente del tejido en el que se encuentran. Los genes alterados no se relacionan con una fase concreta del ciclo celular, sino que están implicados en varias de ellas. En el cerebro, por ejemplo, destacan los genes implicados en la fase G_1, en la transición G_1/S y en los puntos de control del ciclo y arresto celular.

Entre los genes que se expresan más tanto en cerebro como en CMSPs en APP/PS1 frente a controles encontramos *Nek2* (*NIMA(never in mitosis gene a)-related Expressed Kinase 2)*, *Cdk5rap1 (CDK5 Regulatory subunit-Associated Protein 1)* y *Cdkn2a (CDK inhibitor 2a).* Nek2 es un miembro de la familia de serina-treonina quinasas Nek, que se relaciona estructuralmente con el regulador de la mitosis NIMA (Fry y cols., 1998). Nek2 está implicada en la división celular y en la regulación mitótica a través de la separación del centrosoma (Fry, 2002; Fletcher y cols., 2004). Esta proteína también se ha encontrado elevada en diversos tipos de cáncer (Hayward y cols., 2004) y se ha descrito que su sobreexpresión es capaz de promover la aneuploidía en algunos tipos celulares (Liu y cols., 2010; Zeng y cols., 2010) . El gen *Cdk5rap1* codifica una proteína asociada con la subunidad reguladora de Cdk5. En muchos modelos celulares se ha descrito la correlación entre la desregulación de Cdk5 y la muerte neuronal. Además de ser una de las principales quinasas que intervienen en la hiperfosforilación de tau, Cdk5 también interviene en la muerte neuronal producida por la reentrada en ciclo ya que induce la fosforilación de la proteína del retinoblastoma (Hamdane y cols., 2005; Hamdane y Buee, 2007). Por su parte, los resultados del array de PCR muestran que el gen *Cdkn2a*, que codifica el inhibidor de Cdks p16, presenta una expresión más de tres veces superior en el cerebro de los ratones transgénicos que en el de los controles. Este resultado está de acuerdo con otros publicados con anterioridad, en los que se observaba una sobreexpresión de p16 en neuronas de pacientes de EA (Arendt y cols., 1996; McShea y cols., 1997). En cuanto a los genes que se expresan en menor medida en ratones transgéni-

cos frente a controles en ambos tejidos, podemos destacar *Cdkn1a*, que codifica p21. En la corteza de pacientes de EA ya se habían descrito con anterioridad alteraciones en la expresión de este inhibidor del ciclo celular (Engidawork y cols., 2001; Zhu y cols., 2004a). Asimismo, de acuerdo con estos resultados, en nuestro laboratorio se había descrito una disminución de los niveles de p21 en linfoblastos de pacientes de EA respecto a controles en condiciones proliferativas (Sala y cols., 2008). La expresión de *Gpr132 (G Protein-coupled Receptor 132),* por su parte, está disminuida en el cerebro y aumentada en las CMSPs de los ratones APP/PS1 respecto a los controles, lo que puede tener distintas implicaciones funcionales. El receptor que codifica este gen se identificó en células linfoides B como el receptor de lisofosfatidilcolina (Kabarowski y cols., 2001) debido a que su transcripción aumentaba en respuesta a la transformación oncogénica o tras el tratamiento con agentes que causan daños en el ADN (Weng y cols., 1998). En resumen, los resultados del array de PCR muestran que existe un buen número de genes relacionados con el ciclo celular cuya expresión está alterada tanto en el cerebro como en los linfocitos del modelo transgénico de alzhéimer APP/PS1.

En consonancia con los cambios observados en la expresión de genes que controlan el ciclo celular en los CMSPs del ratón transgénico, también observamos una mayor tasa de incorporación de BrdU en el ADN en los ratones APP/PS1 respecto a los controles. Este resultado refleja una mayor actividad proliferativa en respuesta a un estímulo mitogénico de los CMSPs de los ratones transgénicos respecto a los controles, y además está de acuerdo con los resultados previos de nuestro laboratorio, en los que mostrábamos una mayor proliferación tanto de CMSPs como de linfocitos inmortalizados de pacientes de EA respecto a individuos sanos (de las Cuevas y cols., 2003; Bartolomé y cols., 2007; Muñoz y cols., 2008).

Por otra parte, las alteraciones en genes del ciclo celular en el cerebro de ratones APP/PS1 se acompañan de la expresión de un marcador de proliferación como PCNA o de la proteína inhibidora de Cdks, p16, en neuronas postmitóticas. Estas observaciones sugieren que la sobreexpresión de APP y la mutación en *PS1* pueden inducir a las neuronas diferenciadas a entrar en un ciclo de división no deseado. Nuestros resultados están de acuerdo con publicaciones previas en las que se describen aumentos en la expresión de distintas proteínas reguladoras del ciclo en el cerebro de pacientes de la enfermedad (Nagy y cols., 1997; Busser y cols., 1998; Schmetsdorf y cols., 2007) o en el de varios modelos murinos de EA (Yang y cols., 2006; Ahn y cols., 2008; Malik y cols., 2008). Sin embargo, también hay que señalar que otros autores no han encontrado evidencias de reactivación del ciclo celular en un modelo triple

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transgénico de la enfermedad (3xTg-AD) (Lopes y cols., 2009a).

A pesar de que son numerosos los resultados que apuntan a la reactivación del ciclo celular en las neuronas en varios modelos murinos de la EA y que también se han descrito características apoptóticas en el ratón APP/PS1 (Spuch y cols., 2010), este modelo, y, en general, la gran mayoría de los modelos de EA, no muestran una pérdida neuronal significativa (Oddo y cols., 2003; Yang y cols., 2006), lo que puede hacer pensar que la disfunción del ciclo no sea una causa inmediata de muerte neuronal. En ese sentido, la teoría del doble impacto sugiere que las neuronas afectadas puedan sobrevivir a pesar del intento de entrada de ciclo, pero en un estado que las hace más vulnerables a otros factores nocivos (Zhu y cols., 2004b; Zhu y cols., 2007a).

Además de nuestros resultados mostrando alteraciones paralelas en cerebro y células periféricas, previamente se había descrito que tanto los linfocitos como los cultivos neuronales de ratones portadores de una o más mutaciones en el gen de PS1 mostraban una mayor vulnerabilidad celular frente al estrés (Eckert y cols., 2001b). Por lo tanto, nuestras observaciones suponen un apoyo adicional a la idea de que la EA induce también cambios significativos en la fisiología celular a nivel sistémico.

Una limitación importante de nuestro estudio es la heterogeneidad de las muestras de cerebro. En este tipo de muestras, otras células además de las neuronas, como las células de la glía o incluso los progenitores neurales, podrían contribuir a la expresión diferencial de genes relacionados con el ciclo celular en los ratones APP/PS1. Aunque hemos podido demostrar la expresión alterada de PCNA y p16 específicamente en neuronas diferenciadas de la corteza de estos ratones, lo ideal sería confirmar los cambios en la expresión génica en poblaciones específicas de neuronas como las que se obtienen por microdisección mediante captura con láser. En los ratones APP/PS1 hemos observado que, a pesar de presentar una mayor activación glial que los ratones control, la co-localización de p16 y el marcador de astrocitos GFAP es muy escasa.

En resumen, los resultados presentados en este apartado muestran que la sobreexpresión de APP y la mutación de *PS1* inducen en el ratón una serie de alteraciones en el control del ciclo celular que se producen de manera paralela en el cerebro y en las CMSPs, apoyando la existencia de manifestaciones sistémicas de la enfermedad y validando el empleo de tejidos periféricos para el estudio de las características fisiopatológicas de la EA y también para valorar la eficacia de tratamientos contra la enfermedad. Algunos fármacos capaces de detener la progresión del ciclo celular han mostrado efectos neuroprotectores en modelos animales de daños cerebrales o infarto (Woods

y cols., 2007; Varvel y cols., 2009) y en la actualidad están en marcha diversos estudios basados en la hipótesis del ciclo celular.

Además de lo expuesto anteriormente, los modelos animales de la EA también se emplean en la actualidad para la búsqueda de biomarcadores. Se entiende por biomarcador un parámetro molecular, bioquímico o anatómico que puede ser cuantificable y que es característico de un determinado estado de salud o enfermedad. En el caso de la EA, los biomarcadores pueden ser útiles en el diagnóstico temprano de la enfermedad, en el estudio de la progresión de la misma o para evaluar la eficacia de potenciales tratamientos terapéuticos. Lo ideal en este tipo de indicadores es que sean de fácil obtención, y, dadas las características de la EA, que se puedan obtener empleando técnicas no invasivas. En ese sentido, destacan la búsqueda de biomarcadores en fluidos corporales y el estudio anatómico, funcional y metabólico del cerebro mediante técnicas de resonancia magnética. Dentro de estas últimas, tanto la resonancia magnética de imagen (MRI) como de espectroscopía (MRS), son de especial interés y se están empleando en la actualidad tanto en humanos como en modelos animales (Bradley y cols., 2002; de Leon y cols., 2007; Leow y cols., 2009). Es importante hacer notar que los biomarcadores encontrados en humanos no son necesariamente extrapolables a los que podemos encontrar en modelos animales (y viceversa) por lo que es interesante caracterizar los modelos animales en la búsqueda de los parámetros que mejor definan al

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modelo en cuestión.

En general, el uso de MRI y MRS en los modelos de ratón se ha centrado en el estudio de la aparición de placas de amiloide (Lee y cols., 2004; Wengenack y cols., 2008), en los cambios volumétricos (Lau y cols., 2008; Van Broeck y cols., 2008) y en la cuantificación de metabolitos por MRS (Marjanska y cols., 2005; von Kienlin y cols., 2005). En nuestro caso, el abordaje ha sido diferente, y hemos estudiado de manera general la totalidad del cerebro del ratón, así como estructuras concretas (hipocampo y corteza, las zonas más afectadas por la enfermedad). El objetivo principal de este bloque de la tesis ha sido caracterizar sistemáticamente por técnicas de MRI y MRS el modelo transgénico APP/PS1, evaluando una amplia variedad de parámetros. Además, hemos aplicado un modelo de regresión logística para determinar, entre todas las variables obtenidas, aquellas que mejor discriminan entre los ratones transgénicos y los controles.

En primer lugar, en cuanto a las técnicas de resonancia magnética de imagen, hemos empleado imágenes pesadas en T2, junto con imágenes de resonancia magnética por difusión y por transferencia de magnetización (ver Material y Métodos). En humanos, las técnicas de MRI normalmente se emplean para el estudio estructural y volumétrico del cerebro, especialmente en la detección de la atrofia del lóbulo temporal-medial y también en los cambios concretos de determinadas estructuras, como el hipocampo y los ventrículos (Frisoni y cols., 2010). La inflamación, otra característica importante de la enfermedad, se relaciona con un aumento en el contenido de agua del cerebro, y también se puede visualizar con este tipo de técnicas, reflejándose un aumento en la intensidad de las imágenes pesadas en T2 y en los valores de ADC junto con una disminución del porcentaje de MT. Los resultados presentados muestran que los ratones APP/PS1 presentan estas características, lo que es compatible con los signos de neuroinflamación previamente descritos en este modelo de EA (Spuch y cols., 2010).

El estudio volumétrico de las diferentes áreas del cerebro muestra que los ratones APP/PS1 presentan un mayor volumen ventricular que los ratones control. Este marcador estructural se ha observado también en pacientes de EA, se correlaciona fuertemente con los resultados en los test cognitivos y en algunos estudios longitudinales los cambios se asocian con la progresión de DCL a EA (Thompson y cols., 2004; Nestor y cols., 2008; Ridha y cols., 2008; Evans y cols., 2010). Además, algunos autores han descrito que los cambios volumétricos en los hemisferios cerebrales (como por ejemplo, la dilatación de los ventrículos) se correlacionan mejor con el deterioro cognitivo que la atrofia del lóbulo temporal medial (Jack y cols., 2004). El incremento en el volumen ventricular también se asocia con las placas de amiloide y la

presencia de ovillos neurofibrilares y puede considerarse un marcador adecuado para evaluar la progresión de la enfermedad (Silbert y cols., 2003). Nuestros resultados muestran un incremento significativo en el volumen de los ventrículos de los ratones APP/PS1 que afecta principalmente a los ventrículos laterales. Se han descrito resultados similares en otro modelo de ratón transgénico de EA con mutaciones en tau (Xie y cols., 2010). También hay que mencionar que el patrón de expansión de los ventrículos a lo largo de la vida del ratón es diferente que en humanos, por lo que hay que tener en cuenta en los ratones la dilatación ventricular que ocurre fisiológicamente con el envejecimiento (Chen y cols., 2011).

El ensanchamiento de los ventrículos se acompaña de una disminución discreta del volumen del hipocampo en los ratones APP/PS1. La atrofia del hipocampo, y especialmente la tasa de atrofia del hipocampo, es decir, los cambios de volumen de esta estructura a lo largo del tiempo, son parámetros que se consideran predictores de la progresión de la EA también en personas con DCL (Henneman y cols., 2009; Mouiha y Duchesne, 2011). Sin embargo, los estudios en modelos transgénicos de la enfermedad muestran resultados contradictorios. Mientras algunos modelos como el PDAPP, el APP/PS1 o el rTg4510 muestran un menor volumen del hipocampo (Redwine y cols., 2003; Oberg y cols., 2008; Yang y cols., 2011), otros resultados describen un mayor volumen en el modelo TASTPM (Maheswaran y cols., 2009). Cabe pensar que el trasfondo genético, los diferentes genes expresados en cada modelo, y otros factores como la edad del animal o incluso el método de medida puedan influir en las diferencias encontradas. En conjunto, parece que el estudio del aumento del volumen ventricular, más que la disminución del volumen del hipocampo en los ratones transgénicos modelos de EA tenga potencialmente un interés mayor para el diagnóstico de la enfermedad.

Los mapas de transferencia de magnetización nos ofrecen información sobre la microestructura del cerebro, reflejando posibles alteraciones histológicas. Nuestros resultados muestran una disminución en el porcentaje de transferencia de magnetización, tanto en el total del cerebro como en la corteza cerebral y el hipocampo. Estos resultados están en consonancia con los encontrados en cerebros de pacientes de EA (Hanyu y cols., 2000; Hanyu y cols., 2001; Kabani y cols., 2002; Ropele y cols., 2012). Además, las alteraciones localizadas en el % MT se han asociado con el estado cognitivo tanto en pacientes con DCL como con EA (van Buchem y Tofts, 2000; van der Flier y cols., 2002). La reducción del % MT en el cerebro se puede relacionar con situaciones de inflamación o edema, y algunos autores han sugerido que pueda reflejar cambios relacionados con la pérdida neuronal, la acumulación de placas y ovillos y la gliosis (Ridha y cols., 2007).

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Los valores de ADC, por su parte, nos dan una idea de la difusión del agua en los tejidos. En los cerebros de pacientes de EA y DCL se ha descrito un aumento en los valores de ADC, que algunos autores han atribuido a la pérdida de cuerpos neuronales, axones o dendritas, que puede tener como consecuencia una expansión del espacio extracelular donde el agua puede difundir de manera más rápida aumentando dichos valores (Sandson y cols., 1999; Kantarci y cols., 2005). Nuestros resultados muestran que los ratones APP/PS1 presentan una tendencia a tener valores más altos de ADC tanto en el total del cerebro como en el hipocampo respecto a los controles, sin embargo, este hallazgo no es estadísticamente significativo. Otro modelo transgénico de EA, el TgCRND8, muestra resultados similares (Thiessen y cols., 2010).

Hemos combinado los estudios de MRI con MRS para analizar cuantitativamente cambios metabólicos en el cerebro del ratón *in vivo*. En general, la reducción del pico de NAA es el indicador metabólico más consistente de patología cerebral y de la progresión de la enfermedad en la EA, ya que está relacionado con la degeneración neuronal (Jessen y cols., 2005; Foy y cols., 2011; Silveira de Souza y cols., 2011). Aunque algunos modelos transgénicos de EA también muestran una disminución del cociente NAA/Cr en el hipocampo de ratones entre 6,5 y 9 meses de edad (Oberg y cols., 2008), nosotros no hemos encontrado diferencias en este cociente ni en la corteza ni en el área subcortical de los ratones APP/PS1. Sin embargo, nuestros resultados podrían estar de acuerdo con otras publicaciones en las que se muestra una disminución de NAA/Cr en el cerebro de ratones APP/PS1 pero solamente en aquellos de más de doce meses de edad (Marjanska y cols., 2005). Asimismo, en el modelo transgénico PS2APP los cambios en los niveles de NAA también dependen de la edad y están relacionados con la amiloidosis (von Kienlin y cols., 2005).

Nuestros resultados muestran que los estudios de MRS en el ratón APP/PS1 reflejan un incremento muy significativo en el cociente Cho/Cr en las áreas cortical y subcortical respecto a los ratones control. Otros modelos animales, sin embargo, no reflejan cambios en la concentración de Cho (Marjanska y cols., 2005). Las discrepancias pueden deberse a diferencias en la región cerebral estudiada o a diferencias intrínsecas al modelo transgénico o relativas a su edad. En humanos, hay también resultados conflictivos, mientras que algunos autores encuentran en pacientes de EA un mayor valor del cociente Cho/Cr (Kantarci y cols., 2004), otros no lo hacen (Valenzuela y Sachdev, 2001; Wang y cols., 2009c). Es importante destacar que este cociente permite diferenciar totalmente a los ratones APP/PS1 de los ratones control a los doce meses de edad. Por lo tanto este valor podría considerarse como un marcador muy sensible *in vivo* de la presencia de la enfermedad, al menos a los doce meses de edad. Un estudio longitudinal

prospectivo realizado en 509 personas de edad avanzada mostraba que las personas con un cociente Cho/Cr elevado presentaban más riesgo de desarrollar demencia en los cuatro años siguientes (den Heijer y cols., 2006). Además, recientemente se ha descrito que la elevación de este cociente se asocia con procesos patológicos preclínicos de la EA, de forma que valores Cho/Cr elevados se correlacionan con puntuaciones más bajas en los test cognitivos, y que este proceso es independiente de la carga de Aβ (Kantarci y cols., 2011).

Nuestras observaciones muestran que el incremento en el cociente Cho/Cr en los ratones APP/PS1 respecto a los controles se acompaña de un incremento en la neurogénesis como muestran la incorporación de BrdU y la co-localización del marcador de proliferación Ki-67 y del marcador de precursores neuronales DCX *(Doublecortin).* Algunos modelos de ratón han mostrado alteraciones en la neurogénesis, con resultados contradictorios, mientras unos muestran un aumento en la misma, otros apuntan a una disminución (Donovan y cols., 2006; Lopez-Toledano y Shelanski, 2007). El aumento en la proliferación de los precursores neuronales que nosotros mostramos está de acuerdo con trabajos recientes que indican que la leptina induce una proliferación celular similar en el hipocampo del ratón APP/PS1 adulto (Perez-Gonzalez y cols., 2011), y con resultados post-mortem en el cerebro de pacientes que muestran un aumento de la neurogénesis en el giro dentado y en el área CA1 del hipocampo (Jin y cols., 2004). El incremento en la neurogénesis puede representar una respuesta compensatoria al daño cerebral, aunque sería necesario estudiar si estos progenitores son capaces de diferenciarse en neuronas maduras funcionales que se integren satisfactoriamente en el circuito neuronal.

Otra posible explicación para el incremento en la concentración de Cho tanto en pacientes de EA como en nuestro modelo de ratón es que la colina proceda del catabolismo de fosfatidilcolina de la membrana celular, cuyo objetivo sería proporcionar colina libre para intentar compensar la síntesis deficiente de acetilcolina que tiene lugar en la enfermedad (Wurtman y cols., 1985). Se ha descrito que el incremento de Cho/Cr en el estriado del ratón APP/PS1 se mitiga al tratar a los animales con el inhibidor de acetilcolinesterasa donepezilo (Westman y cols., 2009), un resultado que está de acuerdo con un estudio realizado en pacientes, en los que el cociente Cho/Cr disminuía tras cuatro meses de tratamiento con el fármaco (Bartha y cols., 2008). Estos resultados hacen pensar que el cociente Cho/Cr pueda servir como marcador de la eficacia terapéutica en ensayos clínicos.

El análisis estadístico por regresión logística indica que el parámetro más sensitivo y específico para discriminar entre ratones control y transgénicos a los doce meses de edad es la me-

dida del cociente Cho/Cr en el área subcortical. La combinación de otros parámetros de MRI en la corteza cerebral (Intensidad T2 en el corte 10, % MT en el corte 4 y ADC en el corte 1) también discrimina totalmente ambos grupos. Otros marcadores estructurales, como los cambios volumétricos en los ventrículos y el hipocampo nos dan información importante sobre el estado de enfermedad, aunque con menores valores de especificidad y sensibilidad. En conjunto, estas observaciones ponen de manifiesto la utilidad de los marcadores basados en MRI y MRS en la caracterización de los modelos transgénicos de la EA.

Una importante limitación de nuestro estudio es que solamente se han empleado ratones de doce meses de edad, por lo que los resultados obtenidos de momento sólo se pueden considerar marcadores del estado de enfermedad, lo que los hace útiles para el diagnóstico. Sería muy interesante realizar estudios longitudinales, midiendo los parámetros a diferentes edades para comprobar si estos indicadores pudieran también utilizarse para monitorizar la progresión de la enfermedad.

El modelo de ratón APP/PS1 se había caracterizado previamente en términos de depósito de amiloide, alteraciones del comportamiento, características apoptóticas, y degeneración neuronal y sináptica (Holcomb y cols., 1998; Antequera y cols., 2009; Spuch y cols., 2010). Los estudios de MRI y MRS que hemos realizado añaden más información sobre los parámetros estructurales y bioquímicos que caracterizan al ratón APP/PS1 a los doce meses de edad y pueden ser muy útiles para el estudio de la patología de la EA. Además, existe una gran correlación entre los resultados que presentamos y los que se observan en humanos, por lo que, a nivel translacional podrían ser observaciones muy interesantes en el desarrollo y validación de nuevas estrategias terapéuticas.

Conclusiones

6

1.- Los linfoblastos derivados de pacientes de EA muestran niveles elevados de CaM como consecuencia de alteraciones en la degradación de la molécula en el proteasoma, en un mecanismo dependiente de los niveles de ROS y de calcio intracelular. El aumento de CaM conlleva un incremento en la activación de PI3K y de CaMKII.

2.- Ca2+/CaM interacciona con las vías de señalización PI3K/Akt y ERK1/2 para el control de la proliferación y la supervivencia celular respectivamente, en función de la disponibilidad de factores tróficos. La regulación del destino celular se ejerce a través de cambios en los niveles de dos proteínas clave en el control del ciclo celular: p27 y p21.

3.- Los linfoblastos de EA presentan niveles más elevados de p21 y son más resistentes que las células control a la apoptosis inducida por la retirada de suero. La vulnerabilidad depende de la relación inversa que parece existir entre la actividad de CaMKII y ERK1/2 y entre ésta última y los niveles de p21.

4.- En ausencia de suero, los niveles de p21 parecen ser regulados a nivel transcripcional. En los linfoblastos de EA, la menor activación de ERK1/2 se asocia con la retención nuclear y el aumento de la actividad del factor de transcripción FOXO3a.

5.- Dado que las alteraciones en proteínas reguladoras del ciclo celular y moléculas señalizadoras encontradas en linfocitos de EA son similares a las descritas en neuronas vulnerables de pacientes, estos resultados sugieren que existe una huella molecular específica de la enfermedad a nivel sistémico.

6.- Los ratones APP/PS1 muestran una expresión diferencial de genes relacionados con el ciclo celular en cerebro y en linfocitos, que se acompaña de una mayor actividad proliferativa de linfocitos y de la presencia de marcadores del ciclo celular en neuronas corticales. Estos resultados validan el uso de células periféricas para estudiar los fenómenos relacionados con la neurodegeneración asociada a la reactivación del ciclo celular en neuronas postmitóticas.

7.- La aplicación de técnicas de resonancia magnética de imagen y espectroscopia, MRI y MRS, para el estudio del cerebro de los ratones APP/PS1, ha permitido una caracterización exhaustiva de este modelo experimental, desvelando la potencialidad de algunos de los cambios observados como biomarcadores para ayudar en el diagnóstico y en el desarrollo preclínico de nuevas estrategias terapéuticas.

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Anexos

Se adjuntan a continuación otros artículos relacionados con el trabajo de la tesis que no han sido incluidos en la misma.

- Muñoz, U, Bartolome, F, **Esteras, N**, Bermejo-Pareja, F y Martin-Requero, A (2008). "On the mechanism of inhibition of p27 degradation by 15-deoxy-Delta12,14-prostaglandin J2 in lymphoblasts of Alzheimer's disease patients." Cell Mol Life Sci 65(21): 3507-3519.

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Cellular and Molecular Life Sciences

Research Article

On the mechanism of inhibition of p27 degradation by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 in lymphoblasts of Alzheimer's disease patients

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Abstract. It has been proposed that neuroinflammation, among other factors, may trigger an aberrant neuronal cell cycle re-entry leading to neuronal death. Cell cycle disturbances are also detectable in peripheral cells from Alzheimer's disease (AD) patients. We previously reported that the anti-inflammatory 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) increased the cellular content of the cyclin-dependent kinase inhibitor p27, in lymphoblasts from AD patients. This work aimed at elucidating the mechanisms of 15d-PGJ_2-induced p27 accumulation. Phosphorylation, half-life, and the nucleo-cytoplasmic traffic of p27 protein were altered by 15d-PGJ_2 by mechanisms dependent on PI3K/Akt activity. 15d-PGJ_2 prevents the calmodulin-dependent Akt overactivation in AD lymphoblasts by blocking its binding to the 85-kDa regulatory subunit of PI3K. These effects of 15d-PGJ_2 were not mimicked by 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, suggesting that 15d-PGJ_2 acts independently of peroxisome proliferator-activated receptor γ activation and that the α,β-unsaturated carbonyl group in the cyclopentenone ring of 15d-PGJ_2 is a requisite for the observed effects.

Keywords. Alzheimer's disease, lymphocytes, cell proliferation, p27, proteasome activity, PI3K/Akt, cyclopentenone prostaglandins, Ca^{2+}/calmodulin.

Introduction

Alzheimer's disease (AD) is a complex disorder afflicting the rapidly growing elderly segment of today's population, for which the current therapeutic tools offer only moderate symptomatic relief.

The AD brain pathophysiology includes not only the deposition of amyloid-β-protein, and neurofibrillary tangles but also, among other aspects, signs of chronic inflammation associated with significant neuronal loss [1]. Epidemiological studies have shown that long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of developing AD and delays its onset [2]. NSAIDs are known as ligands for the peroxisome proliferator-activated receptor γ (PPAR γ) [3], and it is believed that some of their

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effects are exerted through the activation of this transcription factor. Several natural and synthetic ligands for PPARγ have been described, among them, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), a prostaglandin J_2 metabolite, has been shown to be the most potent activator. 15d-PGJ_2 is known to increase in the course of inflammatory processes [4]. 15d-PGJ_2 displays anti-inflammatory and protective effects against certain types of injury in cellular systems, animal models and humans [5–7]. In addition to these effects, 15d-PGJ_2 has been shown to induce cell growth arrest and apoptosis in several cancer cell types [8, 9].

Research over the last decade has revealed that cell cycle-related events occur in susceptible neurons during AD, and that they are followed by apoptotic cell death [10–12]. Thus, understanding the molecular pathways underlying this cell cycle-mediated neurodegeneration may be important to find new therapeutic targets to slow or prevent the onset and progression of AD [13]. Several factors, including neuroinflammation can trigger aberrant neuronal cell cycle reentry [14]. Therefore, it was hypothesized that the protective effects of NSAIDs in AD could be due not only to their ability to down-regulate the proinflammatory cytokines released by microglia, but also to their anti-proliferative effects [15, 16].

Numerous observations indicate that, while the predominant clinical expression arises from brain pathology, AD has systemic expression at the cellular and molecular levels [17, 18]. Although these alterations appear to have no consequences outside the central nervous system, their parallel expression in the brain could be considered a plausible pathophysiological model to explain partly the clinical manifestations. Of particular relevance to this work is the fact that cell cycle abnormalities are also found in peripheral cells from AD patients [19, 20].

Epstein Barr virus (EBV) infection *in vitro* causes transformation of B cells and generates B lymphoblastoid cell lines (LCLs) [21]. These LCLs retain the phenotype and functions of mature B cells [22]. LCLs have been widely used as models in various biological and medical studies [23]. Previous work from this laboratory, using EBV-immortalized lymphocytes from late-onset AD patients, demonstrated a Ca^{2+}/calmodulin (CaM)-dependent stimulation of cell proliferation and survival of AD lymphoblasts compared with age-matched non-demented donors [24, 25]. Moreover, we reported that immortalization of peripheral lymphocytes from AD patients with the Epstein Barr virus do not alter the cellular response to serum addition or withdrawal [26, 27]. These observations indicate that established lymphoblastoid cell lines could be a suitable model to study the influence of cell cycle-related events in the pathogenesis of AD.

We have previously reported that 15d-PGJ_2 inhibited the serum-enhanced cell proliferation of lymphoblasts from AD patients, by blocking the critical events for G_1/S transition [28]. The cyclopentenone partially inhibited phosphorylation of retinoblastoma protein (pRb) and up-regulated the levels of the cyclin-dependent kinase (CDK) inhibitor p27. This work was undertaken to further study the mechanism(s) involved in 15d-PGJ_2-induced increased levels of p27 protein in AD lymphoblasts. Our results suggest that 15d-PGJ_2 blockade of Ca^{2+}/CaM-mediated overactivation of PI3K/Akt in AD cells is an important part of the mechanism by which the cyclopentenone regulates the expression levels of p27 and cell cycle progression in AD lymphoblasts. We report here that 15d-PGJ_2 appears to impair the binding of CaM to the p85 regulatory subunit of PI3K, thereby decreasing PI3K/Akt activation. This effect of the cyclopentenone results in reduced phosphorylation and degradation, as well as nuclear retention of p27 protein.

Materials and methods

Materials

All components for cell culture were obtained from Invitrogen (Carlsbad, CA). The kinases inhibitors Ly294002, SP600125, PD98059, and 15d-PGJ_2 were obtained from Calbiochem (Darmstadt, Germany). 9,10-Dihydro-15d-PGJ_2 was from Cayman Chemical (Ann Arbor, MI). Radioactive compounds were purchased from Amersham (Uppsala, Sweden). Polyvinylidene fluoride (PVDF) membranes for Western blots were purchased from Bio-Rad (Richmond, CA). Rabbit polyclonal antibodies (pAbs) against human phospho-Akt (Ser473), phospho-ERK1/2, total ERK1/2 and total JNK were obtained from Cell Signaling (Beverly, MA) and phospho-JNK was from Promega (Fitchbury, WI). Mouse monoclonal antibody anti-human lamin B1 (sc-20682) and anti-human PI3K p85α (sc-1637) and pAbs such as rabbit anti-human p27 (sc-528), rabbit anti-human phospho-p27 (Thr187) (sc-16324-R), rabbit anti-human CDK2 (sc-748), rabbit anti-human ubiquitin (sc-913), rabbit anti-human pRb (sc-50), rabbit anti-human SKP2 (sc-7164), rabbit anti-human CaM I (FL-149) and goat anti-human total-Akt (sc-1618) were from Santa Cruz Biotechnologies (Santa Cruz, CA). The enhanced chemiluminescence (ECL) system was from Amersham. MG132 and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide], histone H1, anti-β-actin, anti-α-tubulin antibodies were obtained from

Sigma Aldrich (Alcobendas, Spain). All other reagents were of molecular grade. 15d-PGJ_2 and MG132 were dissolved in dimethyl sulfoxide (Me_2SO). The final concentration of Me_2SO was 0.1 % in the culture medium.

Cell lines

Twenty patients diagnosed in the department of Neurology of the University Hospital Doce de Octubre (Madrid, Spain) with probable Alzheimer according to NINCDS-ADRDA (National Institute of Neurological and Communicative Diseases and Stroke-Alzheimer's Disease and Related Disorders Association) criteria were used in this study. The average age of onset of the disease was 74±2 years. A group of 20 non-demented age-matched individuals was used as control. In all cases, peripheral blood samples were taken after written informed consent of the patients or their relatives.

Establishment of LCLs was performed in our laboratory as previously described [29] by infecting peripheral blood lymphocytes with the EBV [30]. Cells were grown in suspension in T flasks in an upright position, in approximately 10 ml RPMI 1640 (Gibco, BRL) medium that contained 2 mM L-glutamine, 100 µg/ml penicillin/streptomycin and, unless otherwise stated, 10 % (v/v) fetal bovine serum (FBS) and maintained in a humidified 5 % CO_2 incubator at 37°C. Fluid was routinely changed every 2 days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

Determination of cell proliferation

Proliferation was determined either by cell counting in a Neubauer chamber, or by using the MTT assay. This assay is based on the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide by mitochondrial dehydrogenase in viable cells [31]. Potential toxicity of the reagents used was routinely checked by trypan blue exclusion under inverted phase-contrast microscopy.

Cell cycle analysis

Exponentially growing cultures of cell lines were seeded at an initial concentration of 1×10^6 cells/ml. Cell cycle analysis was performed using a standard method [32]. Cells were fixed in 75 % ethanol for 1 h at room temperature. Subsequent centrifugation of the samples was followed by incubation of cells in PBS containing 1 µg/ml of RNase at room temperature for 20 min and staining with propidium iodide (PI; 25 µg/ml). Cells were analyzed in an EPICS-XL cytofluorimeter (Coulter Científica, Móstoles, Spain). Estimates of cell cycle phase distributions were obtained by computer analysis of DNA content distributions.

Proteasome activity assay

The proteasome activity assay was performed in total cell extracts from control and AD lymphoblasts incubated in the absence or in the presence of 1 µM MG132 for 24 h. To measure the proteasome chymotrypsin peptidase activity, 10 µg protein extract was mixed with 300 µl reaction buffer containing 20 mM HEPES, 0.5 mM EDTA pH 8.0, 0.035 % SDS, and 20 µM succinyl-Leu-Leu-Val-Try-7-amido-4-methylcoumarin at 37°C for 90 min. Substrate hydrolysis was measured by monitoring the release of Try-7-amido-4-methylcoumarin using a spectrofluorometer (excitation at 370 nm, emission at 460 nm) as described [33].

Immunological analysis

Cell extracts. To prepare whole cell extracts, cells were harvested, washed in PBS and then lysed in ice-cold lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 50 mM NaF, 1 % Nonidet P-40), containing 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate and protease inhibitor Complete Mini Mixture (Roche, Mannheim).

Cytosolic and nuclear proteins were differentially extracted by lysing cells in ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT) containing 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphatase, and protease inhibitor mixture. After extraction on ice for 15 min, 0.5 % Nonidet P-40 was added, and the lysed cells centrifuged at 4000 rpm for 10 min. Supernatants containing cytosolic proteins were centrifuged at 13 000 rpm for 10 min. Nuclei were washed twice with the hypotonic buffer, and then lysed in hypertonic buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) containing 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate and protease inhibitor mixture. After extraction on ice for 30 min, the samples were centrifuged at 15 000 rpm for 15 min at 4°C. Antibodies to α-tubulin and to lamin B1 were used to assess the purity of the fractions.

The protein content of the extracts was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories).

Western blot analysis. From the whole cell extracts, 50–100-µg samples were fractionated on a SDS-polyacrylamide gel, and transferred to PVDF membrane (Immobilon-P). The amount of protein and the integrity of transfer were verified by staining with

Ponceau-S solution (Sigma). The filters were then blocked with non-fat milk and incubated, overnight at 4°C, with primary antibodies at the following dilutions: 1:500 anti-p27, 1:500 anti-phospho p27, 1:1000 anti-SKP2, 1:500 anti-ubiquitin, 1:1000 anti-phospho Akt, 1:1000 anti-Akt, 1:2000 anti-β-actin, 1:2000 anti-α-tubulin and 1:1000 anti-lamin B1. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Sigma) and detected with a chemiluminescent substrate detection system ECL (Amersham). Blots were stripped and reprobed with anti-β-actin as a protein-loading control. The relative protein levels were determined by scanning the bands with a GS-800 imaging densitometer provided with the Quantity One 4.3.1. software from Bio-Rad, normalized by that of β-actin.

Immunoprecipitation and cyclin E/CDK2 kinase assay

Lymphoblasts from control and AD individuals were seeded at an initial cell density of 1×10^6 cells/ml and incubated for 24 h. Protein extracts (500 µg) were incubated with an antibody against CDK2 for 2 h at 4°C, followed by an incubation with 20 µl protein G-Sepharose for 2 h. Samples were washed in kinase buffer (50 mM KCl, 8 mM $MgCl_2$, 1 mM DTT, 3 mM ATP, 50 mM HEPES, pH 7.4). The immune complexes were resuspended in 40 µl kinase buffer containing histone H1 (0.2 µg/µl). [γ-^{32} P]ATP (10 µCi) was added, and after shaking for 1 h at 37°C, the reaction was stopped by addition of 10 µl 6× SDS sample buffer. After boiling, the samples were resolved by SDS-PAGE on a 12% gel and the phosphorylated histone H1 was visualized by autoradiography. The amounts of histone H1 were detected by gel staining with Coomassie.

Co-immunoprecipitation assays

Lymphoblasts from AD individuals were seeded at an initial cell density of 1×10^6 cells/ml and incubated for 24 h. Protein extracts (600 µg) were subjected to immunoprecipitation overnight at 4°C with an anti-p85 monoclonal antibody in the presence of 0.1 mM $CaCl_2$ or 2 mM EGTA. Samples were incubated then with protein G for 2 h at 4°C. Immunocomplexes were washed three times with ice-cold lysis buffer containing $CaCl_2$ or EGTA, suspended with sample buffer, boiled, resolved by SDS-PAGE, and transferred onto PVDF Immobilon-P transfer membrane filters. Blots were probed with an anti-CaM pAb. Membranes were reprobed with the anti-p85 antibody to check for equal immunoprecipitation efficiency.

Confocal laser scanning microscopy

Lymphoblasts from AD individuals were seeded at an initial density of 1×10^6 cells/ml and incubated for 24 h in the absence or in the presence of 2.5 µM 15d-PGJ_2. Cells were harvested, washed in PBS and fixed in freshly prepared 4% paraformaldehyde in PBS for 30 min at room temperature. After two washes with PBS containing 3 µg/ml BSA, cells (approximately 10 000) were plated onto 0.1% poly-L-lysine-coated glass slides and allowed to adhere by spinning at 700 rpm for 7 min. Cells were permeabilized and blocked with PBS containing 0.2% Triton X-100 and 10% normal goat serum (NGS) for 1 h. Cells were incubated overnight with anti-p27 (1:50), washed, and incubated with Alexa Fluor 488-conjugated secondary antibody (1:200) for 1 h at room temperature. Samples were subsequently washed three times in PBS and mounted in Vectashield® Mounting Medium with DAPI (Vector Labs, UK) and visualized on confocal microscope (Leica DMRE2, Heidelberg, Germany).

Statistical analysis

Unless otherwise stated, all data represent means ± SE. Statistical analysis was performed on the Data Desk package (version 4.0) for Macintosh. Statistical significance was estimated by the Student's *t*-test or, when appropriated, by analysis of variance (ANOVA) followed by the Fischer's LSD test for multiple comparisons. Differences were considered significant at a level of $p < 0.05$.

Results

Effects of 15d-PGJ_2 on cell proliferation

It was previously reported that 15d-PGJ_2 has an anti-proliferative effect on lymphoblasts from AD patients associated with altered control of G_1/S transition and accumulation of p27 protein [28]. For this reason, we first tried to elucidate the effects of 15d-PGJ_2 treatment on the phosphorylation status of the pRb and on the kinase activity of cyclin E/CDK2 complex, the key regulators of transition from G_1 phase of the cell cycle to S phase. Figure 1A shows a time course analysis of the effect of 15d-PGJ_2 on rates of proliferation of control and AD lymphoblasts following serum stimulation. It is shown, in agreement with previous results [28], that AD cells proliferate at a higher rate than control cells (Fig. 1A). Addition of 15d-PGJ_2 had no effect on control cells, but significantly reduced the enhanced proliferative activity of AD cells (Fig. 1A). The effect of 15d-PGJ_2 reducing total cell number in AD cultures is not due to increased cell death. A comparative analysis, by flow cytometry, of cell cycle status of control and AD cells following treatment

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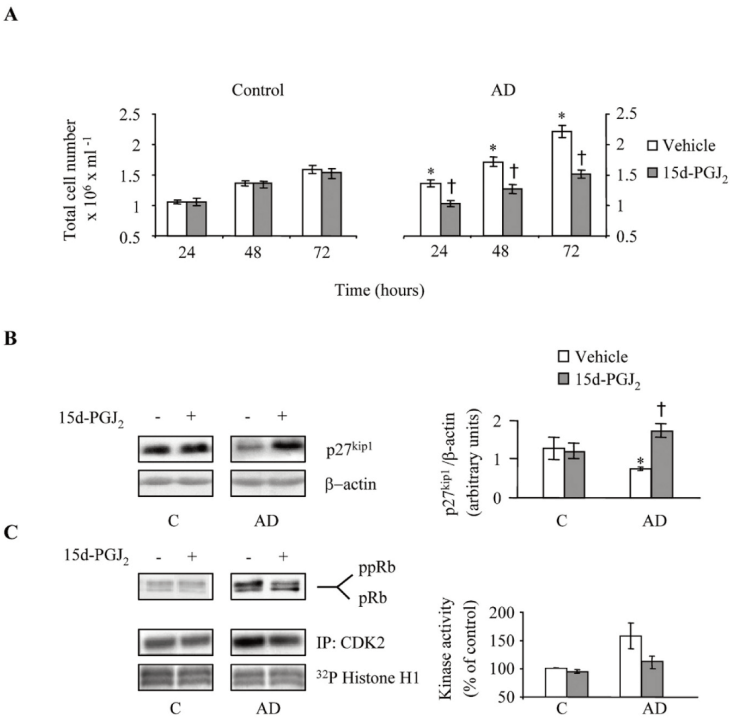

Figure 1. Effects of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) on cell proliferation, p27 content, and phosphorylation status of retinoblastoma protein (pRb), and cyclin E/CDK2 kinase activity in control and lymphoblasts from Alzheimer's disease (AD) patients. (*A*) Lymphoblasts from control and AD subjects were seeded at an initial density of 1×10^6/ml and incubated for 72 h in the absence or in the presence of 2.5 μM 15d-PGJ_2. Aliquots were taken each day for cell enumeration. Values shown are the mean ± SE for seven to fourteen experiments carried out with cells derived from different individuals. *$p<0.01$ significantly different from control cells, †$p<0.01$ significantly different from untreated AD lymphoblasts. (*B*) Whole cell extracts were prepared at 24 h after seeding, and immunoblotted as described in Materials and methods with anti-p27 antibody. A representative immunoblot is shown. Band intensity was measured and normalized by that of β-actin. *$p<0.05$ significantly different from control cells, †$p<0.05$ significantly different from untreated AD lymphoblasts. (*C*) Hypo- and hyper-phosphorylated pRb (ppRb) levels were determined by Western blot in cell extracts prepared 24 h after serum stimulation. Representative Western blots are presented. Immunoprecipitates obtained with anti-CDK2 antibody were assayed for kinase activity using histone H1 as substrate. Phosphorylated histone H1 was visualized using autoradiography. Levels of histone H1 were determined by gel staining with Coomassie. A representative experiment is shown, while below the densitometric analysis is presented. Data shown represent the mean ± SE for four different experiments.

with 15d-PGJ_2 revealed that at the concentration of the drug used, there was no evidence of apoptosis/necrosis in either control or AD cell lines, since a subdiploid pre G_0/G_1 peak was not detected (not shown). As expected, the 15d-PGJ_2-induced inhibition of cell proliferation was accompanied by an accumulation of the CDKi p27 (Fig. 1B). 15d-PGJ_2 inhibited the serum-mediated enhanced cyclin E/CDK2-associated kinase activity in AD cells, and partially blocked pRb phosphorylation (Fig. 1C). Figure 2A shows that the effect of 15d-PGJ_2 inhibiting cell proliferation in AD lymphoblasts is reversible. For these experiments, lymphoblasts from AD patients were incubated in the absence and in the presence of 15d-PGJ_2 for 24 h, then, the medium was changed and cells were enumerated every day. After 15d-PGJ_2 removal, the kinetics of cell proliferation was back to normal (Fig. 2A). As shown in Figure 2B, p27 levels decreased progressively, returning to basal levels following 15d-PGJ_2 withdrawal.

p27 accumulation is due to increased protein half-life in 15d-PGJ_2-treated cells

To determine if the increase in p27 protein following 15d-PGJ_2 treatment resulted from altered protein half-life, AD cells were incubated with and without 15d-PGJ_2 for 24 h at which time cells were treated with cycloheximide to block *de novo* protein synthesis. p27 levels were then determined by Western blot analysis in cells harvested at various time points after

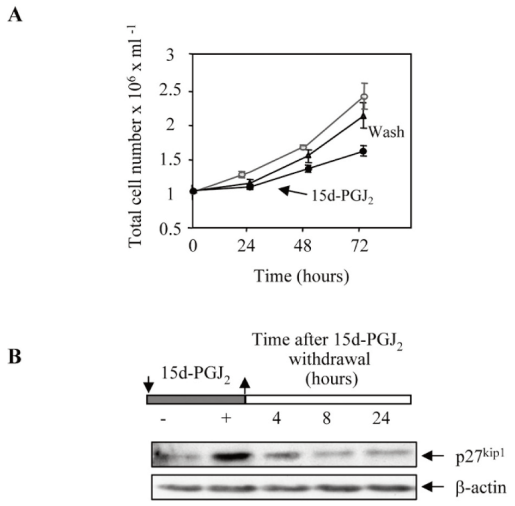

Figure 2. 15d-PGJ$_2$ inhibition of the serum-mediated enhanced proliferation of AD lymphoblasts and increased p27 content are reversible. (*A*) Lymphoblasts from AD patients were incubated in triplicate in 24-well plates (2×10^6/well) in the absence or in the presence of 2.5 μM 15d-PGJ$_2$ for 24 h; the medium was then changed and cells were enumerated every day thereafter. 15d-PGJ$_2$ was kept in one of the wells as control. Data shown are the mean ± SE for four independent experiments. (*B*) Lymphoblasts from AD patients were incubated in the presence of 2.5 μM 15d-PGJ$_2$ for 24 h. After replacing the medium, cells were cultured for an additional period of 24 h. Cells were harvested at the time points indicated in the figure and p27 was detected by immunoblotting. A representative experiment is shown.

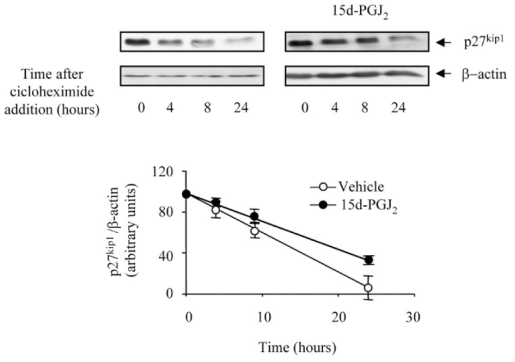

Figure 3. Effect of 15d-PGJ$_2$ on the half-life of p27 protein in AD lymphoblasts. Lymphoblasts from AD subjects were incubated for 24 h in RPMI medium containing 10 % FBS, in the absence or in the presence of 2.5 μM 15d-PGJ$_2$ and then cycloheximide (10 μg/ml) was added. Cells were harvested 4, 8, and 24 h thereafter and p27 was detected by immunoblotting. The decay of the p27 signal is shown as a function of time post-cycloheximide addition. Linear regression curves were fitted to calculate the half-life of the protein using data from four different experiments.

cycloheximide addition. Figure 3 shows that p27 protein from 15d-PGJ$_2$-treated cells persisted longer than from untreated cells. The calculated half-life of p27 increased from 12 h to 22 h after 15d-PGJ$_2$ treatment.

Effects of 15d-PGJ$_2$ on p27 degradation

We next investigated whether the 15d-PGJ$_2$-mediated up-regulation of p27 protein in AD lymphoblasts was due to altered degradation of p27 protein, a process thought to take place in the proteasome [34]. p27 proteolysis is a three-step process that requires phosphorylation at Thr187, recognition by the F-box protein SKP2, ubiquitylation, and degradation by the 26S proteasome [34]. Figure 4 shows that 15d-PGJ$_2$ did not change the phosphorylation status of p27 in control cells, but abrogates the serum-induced increased levels of phospho-p27 (Thr187) in AD cells. There is a tight inverse relationship between levels of phospho-p27 and expression levels (Fig. 4). 15d-PGJ$_2$ treatment had no effect on the SKP2 content in either control or AD lymphoblasts. In addition, we did not find significant differences in the accumulation of ubiquitin-tagged proteins or in total proteasome activity between treated or untreated cells from control and AD patients (Fig. 5). Therefore, these results suggest that, by inhibiting cyclin E/CDK2 kinase activity, and decreasing Thr187 phosphorylation of p27, 15d-PGJ$_2$ abrogates targeting of SCF-ubiquitin E3 ligase and minimizes proteasome degradation of p27, thus increasing cellular content of p27.

Effects of 15d-PGJ$_2$ on PI3K/Akt-mediated down-regulation of p27 in AD lymphoblasts

We have shown previously [26] that serum-mediated enhanced cell proliferation, and decreased levels of p27 in AD cells, requires the activation of PI3K/Akt signaling pathway. Here, we show that the specific inhibitor of PI3K/Akt, Ly294002 mimicked the effects of 15d-PGJ$_2$ inhibiting cell proliferation and increasing the p27 content of AD cells (Fig. 6A and B). When cells were treated with Ly294002 and 15d-PGJ$_2$ at the same time, no further inhibition of cell growth nor increase of p27 were observed (Fig. 6A and B), suggesting no additive effects of these two drugs. In contrast to 15d-PGJ$_2$, the treatment of control cells with Ly294002 inhibited cell proliferation and increased the cellular content of p27 (Fig. 6A and B). The role of 15d-PGJ$_2$ on PI3K/Akt signaling pathway in control and AD lymphoblasts was assessed by monitoring the phosphorylation status of Akt, before and after 15d-PGJ$_2$ treatment, by Western blotting with a phospho-specific anti-Akt antibody. Figure 6C shows significant higher levels of phospho-Akt (Ser473) in AD cells compared with cells from age-

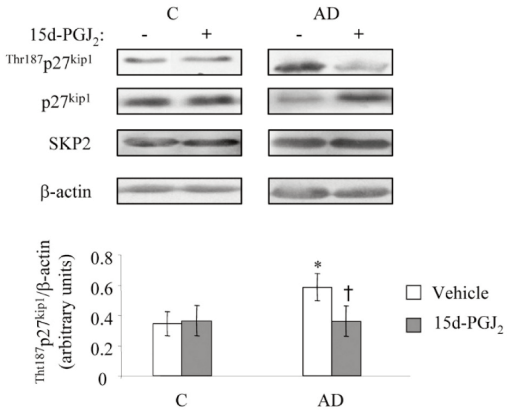

Figure 4. Effects of 15d-PGJ_2 on ^{Thr187}p27 levels, and total p27 and SKP2 content in lymphoblasts from control and AD patients. Lymphoblasts were incubated in RPMI medium containing 10 % FBS in the absence or in the presence of 2.5 μM 15d-PGJ_2 for 24 h. Whole cell extracts were immunoblotted with anti-phospho-p27 (Thr187) and total p27, or with anti-SKP2. Representative immunoblots are presented. Densitometric data for ^{Thr187}p27 protein are the mean ± SE for five experiments. *p<0.05 significantly different from control cells, †p<0.05 significantly different from AD cells incubated in the absence of 15d-PGJ_2.

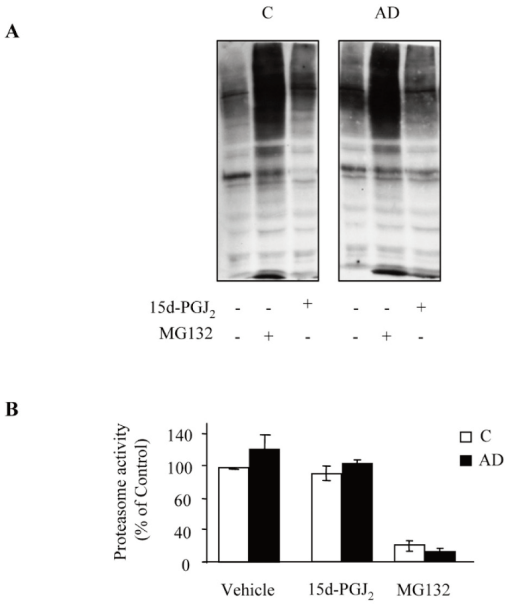

Figure 5. Effects of 15d-PGJ_2 on proteasome activity in control and AD lymphoblasts. (*A*) Lymphoblasts from control and AD patients were cultured for 48 h in the absence or in the presence of 2.5 μM 15d-PGJ_2 or 1 μM MG132 as positive control. Cells extracts were then subjected to immunoblot analysis using an antibody anti-ubiquitin. A representative experiment is shown. (*B*) Cell extracts were assayed for proteasome activity as described in Methods. Data shown represent the mean ± SE of six experiments carried out in cell lines from separate individuals in triplicate.

matched control individuals. Total content of Akt did not change. 15d-PGJ_2 prevented the serum-enhanced Akt phosphorylation in AD cells without affecting Akt activation in control cells (Fig. 6C). These results suggest that 15d-PGJ_2 prevents, rather than inhibits, overactivation of PI3K/Akt pathway in AD cells. Figure 6D shows that 15d-PGJ_2 did not affect either ERK1/2 or JNK activation in lymphoblasts from AD patients.

It has been shown that PI3K/Akt may also contribute to regulation of p27 content by altering the nucleo-cytoplasmic traffic of the protein [35, 36]. For these reasons, we studied whether 15d-PGJ_2 treatment induced changes in the subcellular localization of p27 in AD cells. To this end, AD lymphoblasts were incubated with either 15d-PGJ_2 or Ly294002 and either fractionated to enrich for nuclear or cytoplasmic proteins or processed for immunostaining and confocal laser scanning microscopy. The Western blotting analysis of cytoplasmic and nuclear proteins revealed that 15d-PGJ_2 induced an increase in the nuclear p27 content (Fig. 7A). Similar results were found when AD cells were treated with the PI3K/Akt inhibitor Ly294002 (Fig. 7A). The results of protein fractionation and immunoblotting were consistent with the effects of 15d-PGJ_2 in the localization of p27 determined by immunostaining (Fig. 7B).

Effects of 15d-PGJ_2 on interaction of CaM and PI3K

The fact that 15d-PGJ_2 affects neither Akt phosphorylation nor p27 content in control cells suggests that the cyclopentenone is acting upstream of Akt. 15d-PGJ_2 somehow prevents the serum-mediated enhanced PI3K/Akt pathway in AD cells. Overactivation of PI3K/Akt signaling pathway in AD cells depends on Ca^{2+}/CaM [26]. Moreover, it had been reported that CaM is able to associate with SH_2 domains in the 85-kDa regulatory subunit of PI3K (p85), thereby activating PI3K *in vitro,* and in intact cells [37, 38]. For this reason, we next explored whether 15d-PGJ_2 is able to blunt the potential interaction between PI3K and CaM. To this end, cell lysates from AD lymphoblasts were immunoprecipitated with the anti-p85 antibody. The immunoprecipitates were analyzed by Western blot, and the immunoblots were probed with an anti-CaM antibody. CaM was found to co-immunoprecipitate with p85 (Fig. 8A). As previously described [38], CaM and p85 interaction was found to be Ca^{2+} dependent (Fig. 8A). When 15d-PGJ_2 was added during the immunoprecipitation process, the association was strongly reduced,

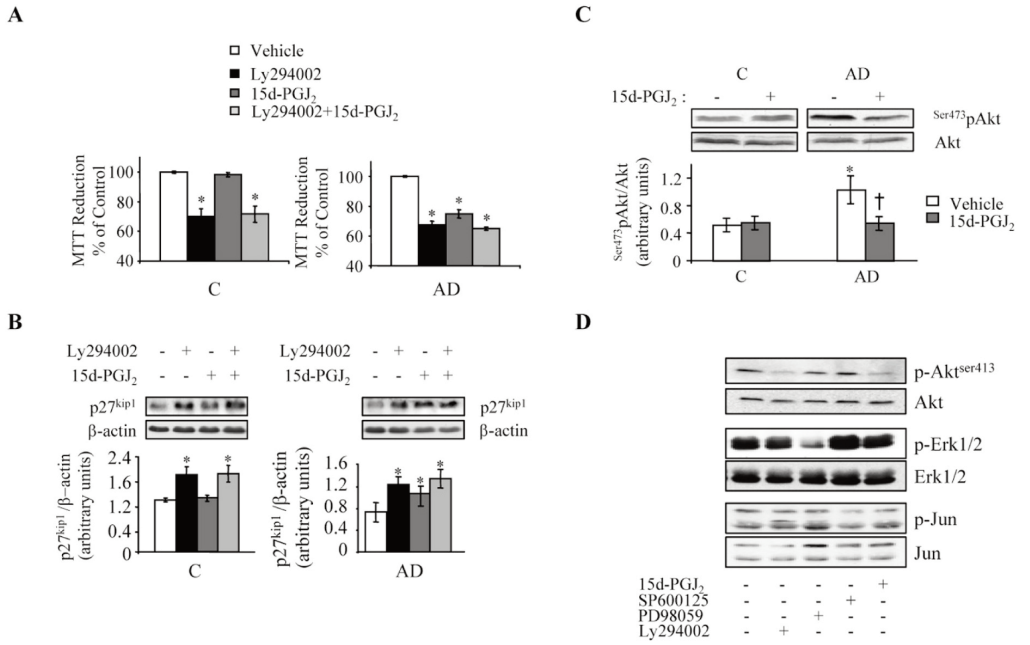

Figure 6. Effects of 15d-PGJ_2 on cell proliferation, p27 content and PI3K/Akt activation in control and lymphoblasts from AD patients. (*A*) Serum-deprived lymphoblasts from control and AD patients were preincubated for 30 min in the presence of 10 μM Ly294002 or 2.5 μM 15d-PGJ_2, and then stimulated by adding 10% FBS. Cell proliferation was assessed, after 24 h, by measuring the MTT reduction. Independent experiments with different cell lines were carried out in duplicate. Values shown are the mean ± SE for six experiments, and expressed as percentage of the value of untreated controls. *$p<0.01$ significantly different from untreated cells. (*B*) Whole cell extracts were subjected to immunoblotting. Representative immunoblots are presented. The immunoreactive bands were quantified by densitometric analysis. Results shown below are the mean ± SE of six determinations made in different experiments. *$p< 0.05$ significantly different from untreated cells. (*C*) Cells were incubated as above. Whole cell lysates were immunoblotted with antibodies anti-phospho-Akt (Ser473) and total Akt. The densitometric data represent the mean ± SE for eight independent experiments. *$p<0.05$ significantly different from control cells; †$p<0.05$ significantly different from untreated AD lymphoblasts. (*D*) Lymphoblasts from AD subjects were incubated in the absence or in the presence of 2.5 μM 15d-PGJ_2, 10 μM LY294002; 10 μM SP600125, or 20 μM PD98059 for 24 h. Whole cell extracts were subjected to immunoblotting with phospho-specific anti-Akt, anti-ERK1/2 and anti-JNK. The same membrane was reprobed with antibodies to the corresponding total protein. Representative immunoblots from five experiments are presented.

indicating that 15d-PGJ_2 displaced CaM binding to PI3K *in vitro* (Fig. 8A). Therefore, these results suggest that treatment of AD cells with 15d-PGJ_2 could prevent CaM-mediated activation of PI3K.

The lack of effects of 15d-PGJ_2 in Akt phosphorylation in control cells suggest the existence of a threshold for CaM activation of PI3K. In consonance with this idea, we found increased expression levels of CaM in AD lymphoblasts compared with those observed in control cells (Fig. 8B).

Effects of 15d-PGJ_2 preventing activation of PI3K/Akt and up-regulation of p27 protein are unlikely to be mediated by PPARγ activation

We previously reported [28] that the anti-proliferative effect of 15d-PGJ_2 in human lymphoblasts is not dependent on PPARγ activation since it could not be blocked by the selective PPARγ antagonist GW9662, and other PPARγ ligands did not mimic the effect of 15d-PGJ_2 on proliferation of AD cells. Data in Figure 9 confirm and extend our previous finding, by showing that cell proliferation (Fig. 9A), Akt phosphorylation and p27 levels are not affected in AD cells by 9,10-dihydro-15d-PGJ_2, an analog of 15d-PGJ_2 that was designed to retain PPARγ agonist activity and to be more resistant to metabolism [39] (Fig. 9B). These results also indicate that the α,β-unsaturated carbonyl group in the cyclopentenone ring of 15d-PGJ_2 is a requisite for the effects of 15d-PGJ_2 in AD cells.

Discussion

Prostaglandins are small lipid molecules that regulate numerous processes in the body and their biological effects have been the subject of intense research in

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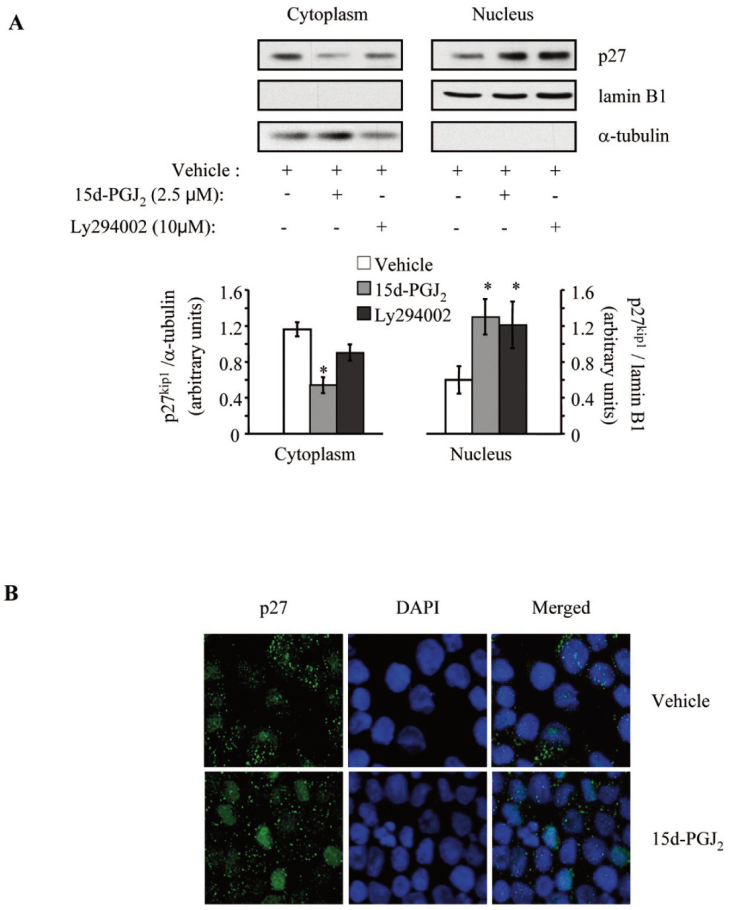

Figure 7. Effect of 15d-PGJ_2 on subcellular localization of p27. (*A*) Lymphoblasts from AD patients were incubated in the absence or in the presence of 2.5 μM 15d-PGJ_2 or 10 μM Ly294002 for 24 h, and fractionated to determine by immunoblot localization of p27. Antibodies to α-tubulin and to lamin B1 were used as control of purity and loading of cytoplasmic and nuclear protein extracts, respectively. Representative immunoblots are shown, whereas the densitometric analysis is presented below. Data represent mean ± SE of eight experiments. *$p<0.01$ significantly different from untreated AD lymphoblasts. (*B*) p27 was studied by confocal laser scanning microscopy. Cells were stained with anti-p27 antibody followed by secondary antibody labeled with Alexa Fluor 488. DAPI was used for nuclear staining. Merged images depict a predominant nuclear localization for p27 in treated cells. Relative intensity was 0.2±0.015 in untreated cells *versus* 1.2±0.07 in the presence of 15 d-PGJ_2. Values are the mean ± SE for 100 cells (magnification 63×).

recent years. The 15d-PGJ_2 is the end-product metabolite of PGD_2 and it is produced by a variety of cells, including mast cells, T cells, platelets, alveolar macrophages, and activated microglia. In monocytes/macrophages, 15d-PGJ_2 exerts an anti-inflammatory action due to the attenuation of the expression of various proinflammatory genes such as IL-1 and TNF, and the expression of effectors proteins such as cyclooxygenase-2, inducible nitric oxide synthase, and matrix metalloproteinases [40–42]. In addition, anti-neoplastic effects of 15d-PGJ_2 have been described in several cell types [8, 9]. Based on these observations, we hypothesized that the protective effects of anti-inflammatory drugs on AD progression could be related to their ability to regulate cell cycle events. It was reported that 15d-PGJ_2 is able to selectively blunt the serum-enhanced proliferation of immortalized lymphocytes from late-onset AD patients without affecting normal proliferative response of control cells [28]. 15d-PGJ_2 inhibits cell cycle progression at the G_1/S checkpoint in AD lymphoblasts [28]. Our results show that treatment of AD cells with 15d-PGJ_2 decreased the activity of the cyclin E/CDK2 complex, apparently by up-regulating the levels of the CDKi p27 and partially blocked the enhanced phosphorylation of pRb protein induced by serum stimulation in AD lymphoblasts. The anti-proliferative effect of 15d-PGJ_2 was shown to be independent of PPARγ activation [28]. In agreement with this observation, we report here the effects of 15d-PGJ_2 are not mimicked by the analog 9,10-dihydro-15d-PGJ_2 that retains PPARγ agonist activity. This finding also indicates that the α,β-unsaturated carbonyl group in the cyclopentenone ring is required for the effects of 15d-PGJ_2 in AD lymphoblasts. PPARγ-independent effects of 15d-PGJ_2 have been previously reported in other cell types, including cells of the nervous system [43, 44]. Several mechanisms have been implicated as

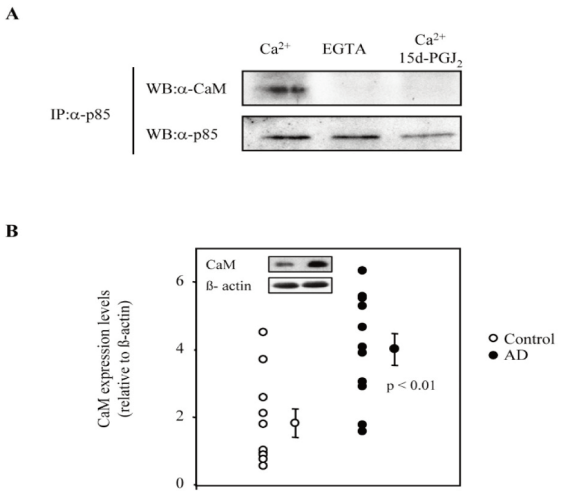

Figure 8. Effect of 15d-PGJ$_2$ on calmodulin (CaM) binding to p85 regulatory subunit of PI3K. (*A*) Lysates from AD lymphoblasts were immunoprecipitated with the anti-p85 antibody (α-p85) in the presence of 0.1 mM $CaCl_2$ or 2 mM EGTA, and 0.1 mM $CaCl_2$ plus 2.5 μM 15d-PGJ$_2$. Immunocomplexes were analyzed by Western blot with an anti-CaM antibody (α-CaM). Efficiency of p85 immunoprecipitation in the different conditions was checked by reprobing the membranes using the anti-α-p85 antibody. The experiment was repeated three times with similar results. (*B*) Scatter plot comparing CaM levels between control (open symbols) and AD lymphoblasts (filled symbols). Cells were seeded at an initial density of 1×10^6/ml and cultured for 3 days in RPMI medium containing 10% FBS. Whole cell extracts were prepared thereafter, and immunoblotted with anti-α−CaM antibody. A representative immunoblot is shown in the inset. Band intensities were measure and normalized by that of β-actin. Statistical significance was determined by the *t*-test.

being responsible for the effects of 15d-PGJ$_2$ on the different cell types and these may partially explain its anti-neoplastic properties. Mediators of these actions include NF-κB inactivation [45], AP1 [38], reactive oxygen species [46], and the MAPK or PI3K/Akt pathways [47, 48]. It has been reported that the PPARγ-independent actions of 15d-PGJ$_2$ may be due to the reactive ring system [39]. Reduction of its double bound may impair significantly the ability of reacting covalently with nucleophile residues in cellular proteins.

This study demonstrates that 15d-PGJ$_2$ blockade of PI3K/Akt overactivation is an important part of the mechanism by which the cyclopentenone regulates the expression levels of p27 and cell cycle progression in AD lymphoblasts. This asseveration finds support in the following observations: first, inhibition of PI3K/Akt by Ly294002 had effects similar to those of 15d-PGJ$_2$ on p27 content and cell proliferation, and second 15d-PGJ$_2$ reduced Akt activation in AD lymphoblasts. Activation of PI3K/Akt pathway has been previously implicated in down-regulation of p27 levels in a number of cell types [49–51] The molecular mechanisms involved in PI3K/Akt-mediated p27 regulation are not completely understood, but include modulation of transcription, protein degradation and subcellular localization of p27. Our data reveal that levels of p27 were post-translationally up-regulated by 15d-PGJ$_2$ by decreasing the rate of p27 degradation in the ubiquitin-proteasome pathway. The half-life of p27 after 15d-PGJ$_2$ treatment of AD cells is considerably lengthened, approaching values observed in lymphoblasts from control individuals [26]. The 15d-PGJ$_2$-mediated up-regulation of p27 protein correlated with inhibition of phosphorylation of p27 at Thr187 by the cyclin E/CDK2 complex, together with increased levels of p27 in the nucleus. The p27-specific F-box, protein SKP2, interacts with the C terminus of the phosphorylated protein in the cytosol. This association results in recruitment of p27 to the SCF core complex, thereby promoting its ubiquitination and degradation [52, 53]. Treatment of AD cells with 15d-PGJ$_2$ did not affect the expression levels of SKP2, the global proteasome activity or the accumulation of ubiquitin-tagged protein. These observations are in line with a recent report showing no differences in peripheral proteasome activity between control and AD lymphocytes [54].

The fact that 15d-PGJ$_2$ affects neither Akt phosphorylation nor p27 content in control cells suggests that the cyclopentenone is acting upstream of Akt. 15d-PGJ$_2$ somehow prevents the serum-mediated enhanced PI3K/Akt pathway in AD cells. We have recently reported that serum-mediated Akt activation in lymphoblasts from AD cells is Ca^{2+}/CaM sensitive [26]. We report here that 15d-PGJ$_2$ is able to impair the binding of CaM to the 85-kDa regulatory subunit of PI3K (p85) *in vitro*. This mechanism could account for the decrease in Akt activation induced by 15d-PGJ$_2$ in AD cells, as it was previously demonstrated that CaM association with SH_2 domains in p85 leads to PI3K activation either *in vitro* or in intact cells [37, 38]. In addition, our results suggest the existence of a threshold for CaM to induce activation of PI3K/Akt pathway in human lymphocytes.

In summary, the present work indicates that 15d-PGJ$_2$ is able to suppress the Ca^{2+}/CaM-enhanced activity of the PI3K/Akt signaling pathway in AD cells, leading to a significant increase in the levels of the CDKi p27, and thus inhibiting cell proliferation (Fig. 10). 15d-PGJ$_2$-mediated decrease of Akt activity impaired the increased phosphorylation of p27 at Thr187 and induced the retention of p27 in the nucleus, thus decreasing the cytosolic degradation of p27 by the proteasome.

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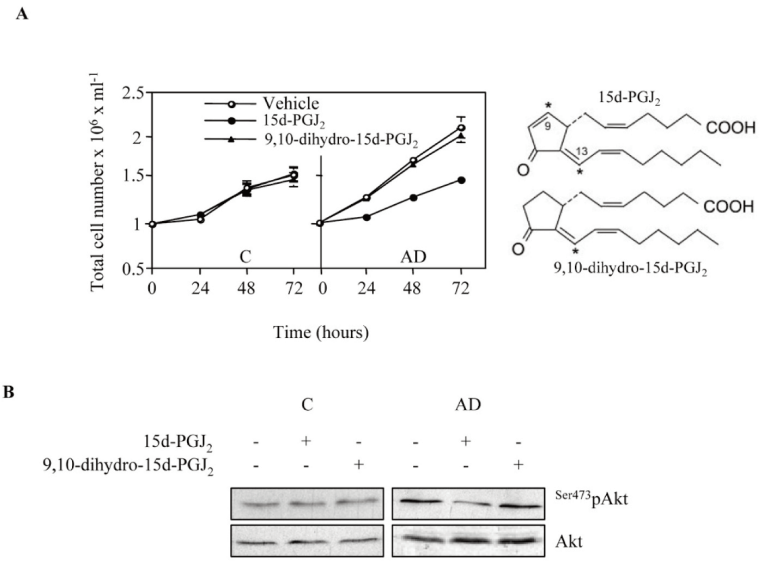

Figure 9. Effects of 15d-PGJ_2 and 9,10 dihydro-15d-PGJ_2 on cell proliferation, p27 content and PI3K/Akt activity in control and AD lymphoblasts. (*A*) Serum-deprived lymphoblasts from control and AD patients were pre-incubated for 30 min in absence or in the presence 2.5 μM 15d-PGJ_2 or 9,10 dihydro-15d-PGJ_2, and then stimulated by adding 10 % FBS and incubated for 72 h. Cells were enumerated each day. Data shown are the mean ± SE for three independent experiments. (*B*) Cells were harvested after 24 h of serum stimulation and p27 was detected by immunoblotting. Densitometric data for p27 are shown below. *$p<0.05$ significantly different from control cells. †$p<0.05$ significantly different from untreated AD lymphoblasts. (*C*) Whole cell lysates were immunoblotted with antibodies anti-phospho-Akt (Ser473) and total Akt. A representative immunoblot is shown.

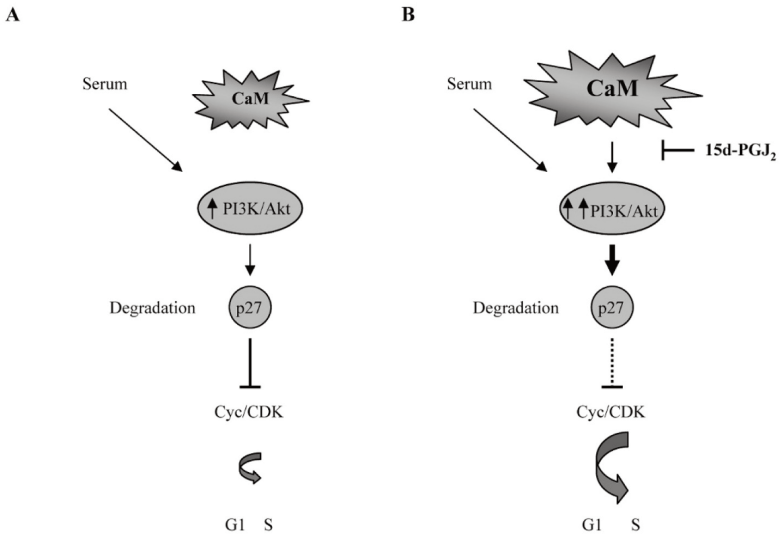

Figure 10. Diagram summarizing the role of 15d-PGJ_2 in preventing Ca^{2+}/CaM-mediated over activation of PI3K/Akt in AD lymphoblasts. (*A*) In control cells, serum stimulation promotes proliferation by inducing PI3K/Akt activity, which in turn, induced p27 degradation. (*B*) In AD cells, in absence of 15d-PGJ_2 increased levels of CaM synergy with serum stimulation and promote overactivation of PI3K/Akt leading to enhanced p27 degradation. Treatment of cells with 15d-PGJ_2 prevent CaM-dependent activation of PI3K/Akt, normalize p27 levels and blocked the serum-mediated enhanced proliferation.

Anexos

Alterations in Akt activity [55], changes in the abundance of p27 [56], as well as changes in cyclin/CDK activity [57] have also been detected in AD brain, suggesting that peripheral cells from patients may be a potential useful surrogate for diagnosis, prognosis and therapeutic monitoring of AD. The alteration in both cyclin/CDK activity and CDKi content seems to contribute significantly to AD pathology since abnormal cyclin/CDK activity appears to correlate with enhanced tau phosphorylation and tangle formation [57]. Moreover, phosphorylated p27 (Thr187) shows considerable overlap with tau-positive neurofibrillary pathology in AD brains [58]. Although cyclopentenone prostaglandins cannot be considered inert compounds, our observation that 15d-PGJ$_2$ had no detectable effects in cell proliferation and signaling thought PI3K/Akt in control cells, while it is capable of selectively suppress the serum-mediated enhanced activation of AD cells, indicates its potential benefit in a therapeutic setting.

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Original Article

Distinct Regulation of Cell Cycle and Survival in Lymphocytes from Patients with Alzheimer's Disease and Amyotrophic Lateral Sclerosis

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Abstract: Alterations in cell cycle progression seem to be associated with neuronal death in Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS). We previously reported disturbances in the control of cell survival/death fate in immortalized lymphocytes from AD patients. These cell cycle dysfunction and impaired apoptosis were considered systemic manifestations of AD disease. The purpose of this study was to evaluate whether these abnormalities are characteristic of AD, or they may be seen in other neurodegenerative disorders such ALS. Our results indicate that alterations in signaling molecules, Akt and ERK1/2, and in the cyclin-dependent kinase complex inhibitors (CDKis) $p21^{Cip1}$ and $p27^{Kip1}$ are detectable in lymphoblasts from AD patients, but not in ALS patients, suggesting that these variables may be considered for the development of biomarkers of AD. However, lymphocytes from ALS patients do not represent a useful model to study cell cycle-related events associated with neurodegeneration of motoneurons.

Keywords: Alzheimer's disease, amyotrophic lateral sclerosis, lymphocytes, cell proliferation, apoptosis, p21, p27, PI3K/Akt, ERK1/2

Introduction

Neuronal death is involved in the onset of irreversible manifestations of Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS). In AD brain, neuronal loss occurs in the memory system of the association cortex, whereas in ALS the upper and lower motor neurons are affected. Although AD and ALS differ in important ways, they also have common pathogenic features, including neuroinflammation, and oxidative and mitochondrial dysfunction leading to apoptosis.

There is increasing evidence suggesting that regulatory proteins of cell cycle progression are also involved in the pathogenesis of neurodegenerative disorders and in the apoptotic death of injured neurons. Cell cycle disturbances have been observed in a number of neurological diseases including AD [1, 2] and ALS [3, 4]. In these studies, it has been suggested that cell cycle signaling might affect neuronal death pathway. The cell cycle is associated with the phase specific expression or modification of defined sets of regulatory genes that control proliferation, differentiation or entry into a quiescent state [5]. However, re-entry of quiescent, terminally differentiated neurons into the cell cycle may result in a mitotic catastrophe and cell death [6-9].

Previous work demonstrated that cell cycle regulatory deficit is not only restricted to neurons of AD. It has also been observed in lymphocytes or fibroblasts of AD [10-13], thus providing a useful tool to further study the involvement of cell cycle-related events in the pathogenesis of AD and for the search of treatment strategies. Moreover, lymphocytes from ALS subjects have been shown to exhibit

Anexos

traits of the disease [14]; however, as far as we know the possible dysfunction of cell survival/death mechanisms has not been studied in peripheral cells from ALS subjects.

Lymphoblasts from AD patients exhibit an enhanced stimulation of proliferation and survival compared with that from control individuals [13, 15-17]. The enhanced proliferative activity of the AD cell lines was associated with a high degree of phosphorylation of Akt and downregulation of the inhibitors of the G1-S checkpoint of the cell cycle, p21 and p27, while the increased survival of serum-deprived AD cells was accompanied by diminished ERK1/2 activation. To investigate whether these abnormalities are characteristic of AD or they are common to neurodegenerative disorders, we generated lymphoblastoid cell lines from ALS patients and compared the cellular response to serum to that of AD lymphoblasts.

Our results indicate that alterations in signaling molecules, Akt and ERK1/2, and in the CDKi p21 and p27 are detectable in immortalized lymphocytes of patients with AD, but not patients with ALS, suggesting that these variables may be considered for the development of biomarkers of AD. However, lymphocytes from ALS patients do not represent a useful model to study cell cycle-related events associated with neurodegeneration of motoneurons.

Materials and Methods

Materials

Polyvinylidene fluoride (PVDF) membranes for western blots were purchased from Bio-Rad (Richmond, CA). Rabbit polyclonal antibodies (pAbs) against human phospho-Akt (Ser473), phospho-ERK1/2, total ERK1/2 were obtained from Cell Signaling (Beverly, MA), and pAbs such as rabbit anti-human p27 (sc-528) and p21 (sc-397), and goat anti-human total Akt (sc-1618) were from Santa Cruz Biotechnologies (Santa Cruz, CA). The enhanced chemiluminiscence (ECL) system was from Amersham (Uppsala, Sweden). Tissue culture media and reagents were obtained from Invitrogen (Carlsbad, CA).

Cell Lines

20 patients diagnosed in the Department of Neurology of the University Hospital Doce de Octubre (Madrid, Spain) of probable Alzheimer according to NINCDS-ADRDA (National Institute of Neurological and Communicative Diseases and Stroke-Alzheimer's Disease and Related Disorders Association) criteria [18]. Of the 20 patients, 7 had mild AD (DSM-III-R, Mini Mental State Examination (MMSE) score between 18-24), 5 had moderate AD (MMSE:10-18), and 8 had severe AD (MMSE: <10). 10 patients suffering from ALS (diagnosed based on the revised El Escorial criteria were used in this study [19]. A group of 20 healthy individuals was used as control. A summary of demographic and clinical characteristics of all subjects enrolled in this study is reported in **Table 1**.

Table 1 Summary of study population

	AD	ALS	HC
Age	75±2	63±3	71±2
Age range	59-89	46-79	43-82
Gender			
Male	9	5	8
Female	11	5	12
Total	20	10	20

HC, healthy control individuals, no sign of neurological disease; AD, patients with a diagnosis of probable AD; ALS, patients with a diagnosis of amyotrophic lateral sclerosis. Values are expressed as mean±SE.

All study protocols were approved by the Spanish Council of Higher Research Institutional Review Board and are in accordance with National and European Union Guidelines. In all cases, peripheral blood samples were taken after written informed consent of the patients or their relatives.

Establishment of lymphoblastic cell lines was performed in our laboratory as previously described [20] by infecting peripheral blood lymphocytes with the Epstein Barr virus (EBV) [21]. Cells were grown in suspension in T flasks in an upright position, in approximately 10 ml of RPMI-1640 (Invitrogen) medium that contained 2 mM L-glutamine, 100 µg/ml streptomycin and, unless otherwise stated, 10 % (v/v) fetal bovine serum (FBS) and maintained in a humidified 5% CO_2 incubator at 37°C. Fluid was routinely changed every two days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

Determination of Cell Proliferation

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Proliferation was determined by cell counting in a Neubauer chamber. EBV immortalized lymphocytes from control and AD individuals were seeded at an initial cell concentration of $1x10^6$ cells/ml. Cells were enumerated everyday thereafter. Potential toxicity of the reagents used was routinely checked by trypan blue exclusion under inverted phase-contrast microscopy.

Preparation of Cell Extracts

To prepare whole cell extracts, cells were harvested and washed in PBS and then lysed in ice-cold lysis buffer (20mM Hepes pH 7.9, 25% glycerol, 0.4M NaCl, 50mM NaF, 1mM EDTA, 1mM EGTA, 1mM DTT), containing 1mM sodium orthovanadate, 1mM PMSF, 1mM sodium pyrophosphate and protease inhibitor complete mini mixture (Roche, Mannhein).

Western Blot Analysis

50-100 µg of whole cell extracts were fractionated on a SDS polyacrylamide gel, and transferred to PVDF membrane (Immobilon-P). The amount of protein and the integrity of transfer were verified by staining with Ponceau-S solution (Sigma). The filters were then blocked with non-fat milk and incubated at 4ºC overnight, with primary antibodies from Santa Cruz at the following dilutions: 1:500 anti-p27, 1:500 anti-p21, 1:1000 anti-phospho Akt, 1:1000 anti-Akt, 1:500 anti-phospho ERK1/2, 1:2000 anti-ERK1/2, and 1:2000 anti-actin. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Sigma) and detected with a chemiluminiscent substrate detection system ECL (Amersham). The relative protein levels were determined by scanning the bands with a GS-800 imaging densitometer provided with the Quantity One 4.3.1. software from BioRad.

Statistical Analysis

Unless otherwise stated, all data represents mean±SE. Statistical analysis was performed on the Data Desk package (version 4.0) for Macintosh. Statistical significance was estimated by analysis of variance (ANOVA) followed by the Scheffe test for multiple comparisons. Differences were considered significant at a level of $p<0.05$.

Results

Proliferation of Immortalized Lymphocytes from Control and AD or ALS Patients

Data in **Figure 1** confirms and extends our previous finding [13, 15, 17, 22] by showing that the serum-induced proliferation of lymphoblasts from AD patients is enhanced compared with that of cells from healthy controls. In contrast, no significant differences were observed between control and ALS lymphoblasts (**Figure 1**).

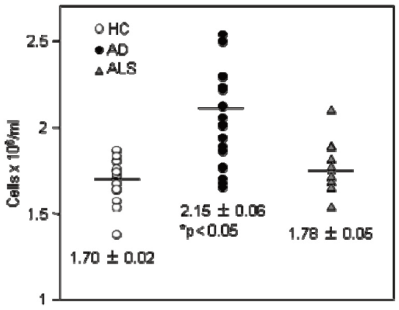

Figure 1 Scatter plot comparing proliferative activity between control and AD or ALS lymphoblasts. Immortalized lymphoblasts from control, AD, and ALS individuals were seeded at an initial density of 1 x 10^6/ml and cultured for 3 days in RPMI medium containing 10% FBS. Everyday thereafter, samples were taken for cell counting. Values shown are from day 3 of culture, and are the mean±SE. Statistical significance was determined by ANOVA.

Since the mean age of ALS patients was lower than that of control or AD subjects, we investigated whether the proliferative activity of control cells was affected by aging. As shown in **Figure 2A**, there were no differences in proliferation of cells derived from subjects younger or older than 70 years old. Moreover, cell proliferation of lymphoblasts from AD patients was not affected by the degree of disease progression, as cell lines from mild, moderate or severe AD patients show similar rates of cell growth (**Figure 2B**).

Cellular Response to Serum Withdrawal in Control and AD or ALS Lymphoblasts

Data in **Figure 3** summarizes the cellular response to serum deprivation of all cell lines used in this study, derived from AD and ALS patients and control individuals. In agreement with previous observations [16], it is shown

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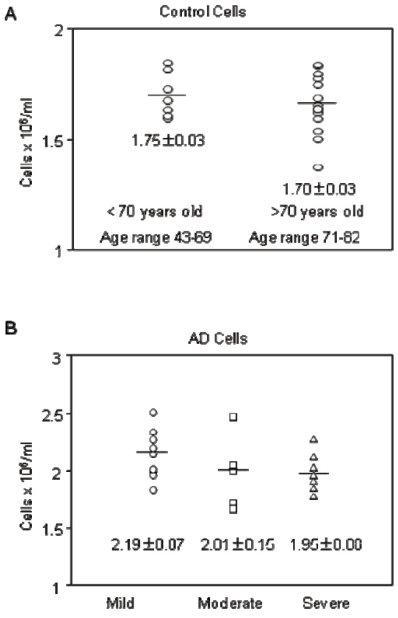

Figure 2 Aging does not affect the proliferative activity of control cells. Neither AD progression influences cell proliferation. **A.** Scatter plot comparing the influence of aging on the proliferation of lymphoblasts from control individuals. Immortalized lymphocytes from control individuals were seeded at an initial density of 1 x 10^6/ml and cultured for 3 days in RPMI medium containing 10% FBS. **B.** lymphoblasts from mild, moderate or severe AD were cultured as above. Values shown are the mean±SE.

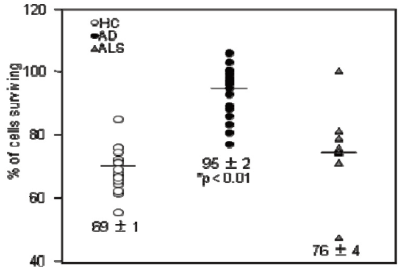

Figure 3 Scatter plot comparing cell survival following serum deprivation between lymphoblasts derived from control, AD or ALS patients. Immortalized lymphocytes from control, AD, and ALS individuals were seeded at an initial density of 1 x 10^6/ml and incubated in serum-free RPMI medium for 72 hours. Cell viability was determined by Trypan blue exclusion under inverted phase-contrast microscopy, and expressed as percentage of the initial number of cells at day 0. Values shown are the mean±SE. Statistical significance was determined by ANOVA.

that AD lymphoblasts were more resistant to serum withdrawal-induced cell death than control cells. The cellular response of ALS lymphoblasts did not differ from that of control cells. In control, as well as in ALS cultures, more than 30% of cells died after 3-day period of serum starvation, whereas less than 10% of AD cells died during the same period of time.

Effect of PI3K/Akt and MAPK Activation on the Survival of Control and AD Lymphoblasts

We have recently reported that alterations of PI3K/Akt and ERK1/2 signaling pathways underlined the abnormal cellular response of AD lymphoblasts to the presence or the absence of trophic support [15, 16]. Enhanced proliferation of AD lymphoblasts was associated with increased activation of PI3K/Akt as monitored by the stimulation of phosphorylation of Akt [15]. Data in **Figure 4** shows, in consonance with the lack of stimulation of proliferation of ALS lymphoblasts, no changes in the cellular content of phosphorylated Akt, compared with levels of control cells. As expected, AD cells show increased phosphorylation of Akt relative to the levels of phospho Akt observed in control or ALS cells (**Figure 4**). PI3K/Akt signaling appears to regulate the G1-S checkpoint of cell cycle by downregulating the levels of two CDK inhibitors p27 and p21 [15, 17]. It was suggested that PI3K/Akt modulates the rate of protein degradation by the proteasome, after phosphorylation of specific residues of these proteins. Therefore, the levels of these proteins were determined in control, AD or ALS lymphoblasts. Only cells from AD patients exhibit significant lower content of p21 and p27 (**Figure 5**).

The ERK1/2 pathway seems to support lethality in immortalized lymphoblasts, as the specific inhibitor PD98059 prevented the serum withdrawal-induced cell death by apoptosis [16]. Moreover, it was shown that deprivation of trophic support induced a sustained increase in the ERK1/2 phosphorylation of significantly lower intensity in AD lymphoblasts [16]. In this work, we have compared the activation of this pathway in

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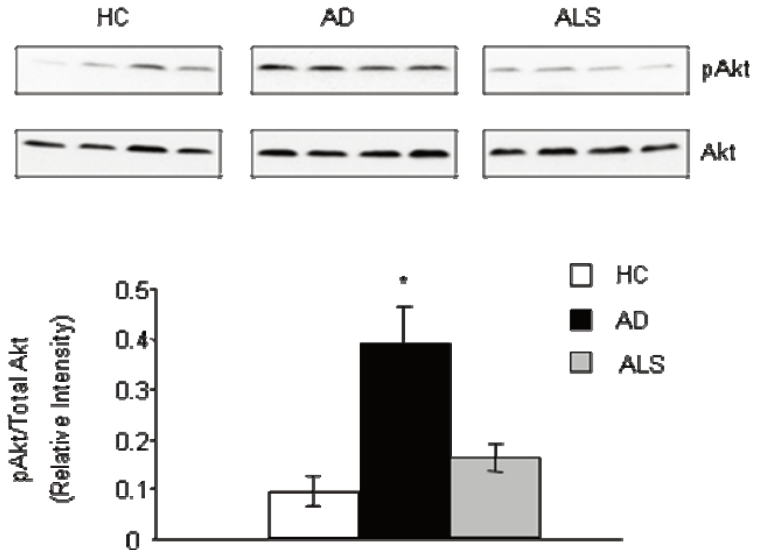

Figure 4 PI3K/Akt activation in control, AD and ALS lymphoblasts. Control, AD, and ALS lymphoblasts from 4 different individuals, were incubated in RPMI containing 10% FBS. Whole cell extracts were prepared 24 h thereafter, and were immunoblotted with antibodies anti-phospho-Akt (Ser473) and total Akt. The densitometric data represent the mean ± SE. *p< 0.05, significantly different from control cells.

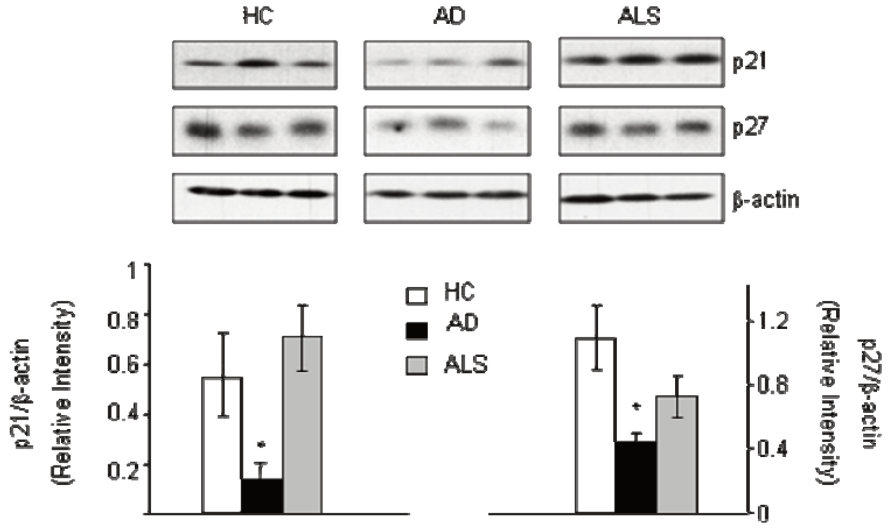

Figure 5 p21 and p27 protein levels in control, AD and ALS lymphoblasts. Lymphoblasts from control, AD or ALS subjects were seeded at an initial density of 1 x 10^6 x ml^{-1} and cultured for 24 h in RPMI medium containing 10% FBS. Thereafter aliquots were taken to prepare cell extracts. Western blots of p21 and p27 proteins in 3 different cell lines from control, AD, and ALS individuals are shown. Densitometric analysis of these proteins are shown below. Results are means ± SE. *p< 0.05 significantly different from control cells.

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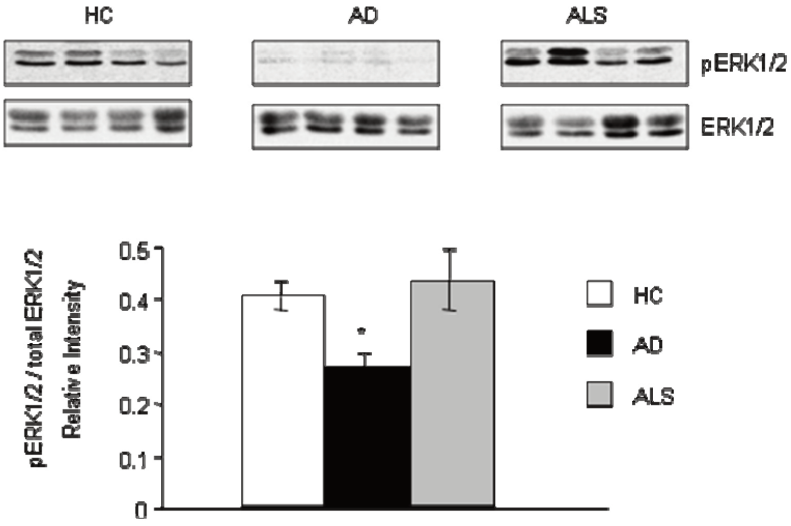

Figure 6 Activity of ERKs in serum deprived control, AD and ALS lymphoblasts. Lymphoblasts from control, AD, and ALS lymphoblasts were serum deprived for 72 h. Thereafter aliquots were taken to prepare cell extracts. The relative levels of activation of p42/p44 ERKs were assessed by western blot analysis using phospho-specific antibodies. The same membranes were then stripped and reprobed with antibodies against total ERKs. Representative Western blots in 4 different cell lines from control, AD and ALS patients are shown. The immunoreactive bands were quantified by densitometric analysis. Results shown below are the mean ± SE of 8 different experiments. *p<0.05 significantly different from control cells.

control, AD and ALS cells 72 hours after serum deprivation. The activity of the ERK1/2 pathway was assessed by western blotting, using phospho-specific antibodies. **Figure 6** shows the state of activation of the ERK1/2 pathway in control and AD or ALS lymphoblasts. As expected, after 72 hours of serum deprivation, the phosphorylation status of ERK1/2 in AD cells is significantly reduced compared with that of control or ALS lymphoblasts. Under these experimental conditions, ALS cells undergo apoptosis as control cells do (**Figure 3**). Thus, these observations suggest that the sustained, but reduced signaling through the ERK1/2 pathway in AD lymphoblasts protects them from the serum withdrawal-induced cell death.

Discussion

Recent work has highlighted the important role of neuronal vulnerability in the instigation and progression of neurodegenerative diseases including AD and ALS [23]. Moreover, a number of published studies indicated that cell cycle status significantly influence neuron vulnerability and neurodegenerative pathways [8, 24-26]. The ability to control cell cycle has been considered a critical factor in preventing neurons entering a vulnerable state with high risk for instigation of neurodegenerative mechanisms [27].

Reports from our and other laboratories had presented evidences indicating that, while the predominant clinical expression arises from the SNC, AD and ALS have systemic expression at the cellular and molecular levels [12, 15, 28-31]. Although these alterations appear to have no clinical consequences outside the central nervous system, their parallel expression in peripheral cells and in the brain, provide a plausible pathophysiological model to explain partly the clinical manifestations. Of particular relevance to this work is the fact that dysfunction of cell cycle is a more general phenomenon affecting cells other than neurons in AD patients [10, 11, 13, 15, 22].

In the present study, we addressed the question whether easily peripheral cells from patients affected with ALS show altered cell

survival/death mechanisms comparable to the previously reported for lymphoblastoid cells lines from AD patients [13-16, 32]. We found that AD cells distinctively proliferate at higher rates and showed a decreased vulnerability to serum deprivation-induced cell death than EBV-immortalized lymphocytes from age-matched control individuals. In addition, we demonstrated that the cellular response to serum addition or deprivation was not affected by the viral transformation [15, 16], indicating that lymphoblastoid cell lines are a useful tool to study the involvement of cell cycle-related events in the neurodegenerative diseases.

Despite the fact that recent literature has shown data regarding systemic manifestation in ALS [30, 33], we did not observed changes in the proliferative capacity of lymphoblasts from ALS patients compared with that of cells from healthy individuals. In contrast, lymphoblasts from AD patients show an enhanced proliferative activity after serum stimulation, in agreement with previous reports from this laboratory [13, 15, 22]. It was reported that overactivation of PI3K/Akt signaling pathway in AD cells, was likely responsible for the enhanced proliferative activity by down-regulating cellular levels of the CDKis, p21 and p27 [15]. In contrast, no significant change in Akt activity was observed in ALS lymphoblasts. Accordingly, p21 and p27 levels were found to decrease only in cells from AD patients. This finding is not in agreement with earlier reports showing deregulation of CDKis associated with neurodegeneration in ALS [4, 34, 35]. A possible explanation for this divergence may be the use of peripheral cells versus motoneurons At present, we cannot ascertain whether the lack of changes in cell cycle regulators in peripheral cells from ALS patients reflects the absence of cell cycle related events associated with neurodegeneration in ALS, or, on the contrary, cell cycle disturbances thought to occur in motoneurons [3], do not have systemic manifestations. In this regard, it is worth to mention a recent report showing no evidence for oxidative stress in fibroblast from ALS patients [36], despite the fact of impaired stress response in affected motoneurons [37].

Other distinct feature of AD cells is to be less vulnerable than control lymphoblasts to cell death induced by serum deprivation [16, 32]. A sustained, but lower, activation of ERK1/2 in AD cells, as compared with the control group seems to protect AD lymphoblasts from death. We report here that lymphoblasts from ALS patients behave as control cells in response to serum withdrawal by showing similar phosphorylation of ERK1/2 and equal susceptibility to the induced cell death.

Collectively, our results show no major alterations in the proliferative capacity or vulnerability to serum deprivation-induced cell death in ALS lymphoblasts compared with cells from control individuals. These observations suggest that these cells do not represent a useful model to study cell cycle-related events associated with motoneurons degeneration.

On the other hand, our findings that cell cycle progression, cell survival and their molecular regulators are distinctly altered in lymphoblasts from AD patients add further support in favor of considering AD as a systemic disease, underlying as possible etiopathogenic mechanism altered responsiveness to cell activation agents. Considering that changes in the abundance of CDK inhibitors had also been detected in AD brain [38], and that perturbation in the activity of PI3K/Akt and ERK1/2 signaling pathways had been detected in AD brains [39-41], our results highlight the usefulness of peripheral cells from AD patients as potential surrogate for diagnosis and therapeutic monitoring of AD.

The cell cycle disturbances and alteration of the apoptotic response found in AD lymphoblasts did not correlate with progression of the disease. They seem to be early manifestations of the disease. This observation is in consonance with recent evidence in AD patients and in animal models [42, 43] indicating that cell cycle dysfunction is an early event in AD pathogenesis. Cell cycle proteins have been found in brains of individuals with mild cognitive impairment (MCI) [42], and cell cycle disturbances have also been reported in lymphocytes from MCI patients [11]. These observations suggest that cell cycle-induced death is a central mechanistic feature of AD, and therefore alterations in cell cycle/apoptosis regulatory proteins may serve as markers of AD disease.

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Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Simvastatin overcomes the resistance to serum withdrawal-induced apoptosis of lymphocytes from Alzheimer's disease patients

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Abstract Statins may exert beneficial effects on Alzheimer's disease (AD) patients. Based on the antineoplastic and apoptotic effects of statins in a number of cell types, we hypothesized that statins may be able to protect neurons by controlling the regulation of cell cycle and/or apoptosis. A growing body of evidence indicates that neurodegeneration involves the cell-cycle activation in postmitotic neurons. Failure of cell-cycle control is not restricted to neurons in AD patients, but occurs in peripheral cells as well. For these reasons, we studied the role of simvastatin (SIM) on cell survival/death in lymphoblasts from AD patients. We report here that SIM induces apoptosis in AD lymphoblasts deprived of serum. SIM interacts with PI3K/Akt and ERK1/2 signaling pathways thereby decreasing the serum withdrawal-enhanced levels of the CDK inhibitor $p21^{Cip1}$ (p21) and restoring the vulnerability of AD cells to trophic factor deprivation.

Keywords Alzheimer's disease · Lymphocytes · Simvastatin · p21 · PI3K/Akt · ERK1/2

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Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme in the conversion of HMG-CoA to mevalonate (MEV), a fatty acid intermediate in the de novo synthesis of cholesterol [1]. Several lipid isoprenoid intermediates such as geranylgeranyl pyrophosphate (GGPP), and farnesyl pyrophposphate (FPP) are also enzymatically generated from MEV through this pathway. These compounds may post-translationally modify small GTP-binding proteins (G proteins), including Rho, Rab, Rac, and Ras, that play pivotal roles in normal and pathological cell signaling [2].

Statins are small-molecule inhibitors of HMG-CoA reductase that effectively reduce low-density lipoprotein cholesterol plasma levels and exhibit additional pleiotropic effects on the vasculature [3, 4]. Therefore, statins are widely used in the prevention and treatment of hypercholesterolemia, atherosclerosis, and cardiovascular and cerebrovascular diseases [5–7]. They have also been associated in some epidemiologic studies with reduced risk of AD [8, 9] and a link between cholesterol and late-onset AD has been documented [10]. However, the evidence from the epidemiological studies on the benefit of statin therapy for AD remains to be solved. Findings from randomized clinical trials evaluating the effects of statins on AD patients had yielded conflicting results [11, 12].

Moreover, a recent population-based study involving 135 elderly people treated with statins and 411 age-matched control individuals living in central Spain, failed to show a benefit in cognition [13]. On the other hand, while some studies examining the role of statins in established AD had shown a potential benefit in cognitive decline [14], a recent large-scale randomized controlled trial evaluating statin therapy for mild to moderate Alzheimer's (the LEADe study) did not find significant benefit on cognition or global function [15]. The beneficial effect of statins beyond lowering cholesterol might, in part, be due to the ability of statins to inhibit the synthesis of FPP and GGPP. It has been recently reported that FPP and GGPP levels are significantly increased in human AD brain [16], suggesting that protein prenylation may contribute to AD pathophysiology. Statins were found to effectively inhibit protein prenylation of a subset of GTPases involved in APP processing, thereby limiting the production of $A\beta_{1-42}$ [17]. On the other hand, it is known that protein prenylation interferes with G protein-mediated cancer survival pathways, and results in the induction of apoptosis in various cancer cells [18, 19]. Therefore, the possible benefit of statins in AD could also be related to the effects of statins modulating cell cycle and apoptosis, as growing evidence suggests that neuronal cell-cycle regulatory failure, leading to apoptosis, may be a significant component of the AD pathogenesis [20, 21].

Aberrant expression of many cell cycle-related proteins and direct evidence for DNA replication in vulnerable neuronal population indicate that, at least, some neurons reentered the cell cycle and entirely passed through a functional interphase having achieved successfully the S phase of cell cycle [22, 23]. However, it is still not clear why neurons then become stuck at the G2 phase, which leads to apoptosis in AD [24]. A number of recent studies have pointed out that in addition to control cell cycle progression by inhibiting the activity of cyclin-dependent kinase complexes (CDKs), p21 can modulate multiple biological functions, including DNA synthesis, stress response, and apoptosis [25]. p21 has been found to be expressed in the cytoplasm of neurons from AD patients [26, 27], often in tangle-bearing neurons and dystrophic neurites, indicating a loss of function as CDK inhibitor and suggesting a possible role of p21 in neuronal apoptosis.

We, and others, have presented evidence that cell-cycle regulatory failure is not restricted to neurons, since peripheral cells from AD patients such as fibroblasts or lymphocytes show altered proliferative activity compared to age-matched control individuals [28–31]. Moreover, we have recently reported that SIM, a lipophilic statin, inhibits cell-cycle progression at the G1-S checkpoint in immortalized lymphocytes from AD patients [32]. These cell lines were also found to be more resistant to serum withdrawal [33]. On these grounds, we found it interesting to study the influence of SIM treatment on the control and AD cell fate upon serum deprivation and in the regulation of cellular content of p21. The results presented here indicate that SIM sensitize AD lymphoblasts to serum deprivation-induced apoptosis. SIM blunted the serum withdrawal-mediated enhanced p21 levels in AD cells by modulating ERK1/2 and PI3K/Akt signaling pathways.

Table 1 Demographic characteristics of all subjects enrolled in the study

	Control ($n = 20$)	AD ($n = 20$)
Age (years)	73 ± 3	74 ± 2
Gender (F/M)	11/9	5/15
Duration of dementia (years)	–	3.6 ± 3
ApoE 4/3 (No. of cases)	1	6

This diagnosis was made according to the criteria developed by the National Institutes of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ARDA). Control individuals with no sign of cognitive disorders. Values are expressed as mean ± SE

AD patients with a diagnosis of probable AD, *F* female, *M* male, *n* number of patients

Materials and methods

Materials

All components for cell culture were obtained from Invitrogen (Barcelona, Spain). The kinase inhibitors PD98059, Ly294002, and the caspase inhibitor benzyloxy-carbonyl-Val-Asp-fluoromethylketone (z-VAD-fmk) were obtained from Calbiochem (Darmstadt, Germany). Poly (vinylidene) fluoride (PVDF) membranes for Western blots were purchased from Bio-Rad (Richmond, CA). Rabbit polyclonal antibodies against human phospho-Akt (Ser473), phospho-ERK1/2(Thr202/Tyr204), total ERK1/2, and goat polyclonal anti total Akt were obtained from Cell Signaling (Beverly, MA USA). Rabbit-anti human p21 antibody (sc-397) was from Santa Cruz Biotechnologies (Santa Cruz, CA). The enhanced chemiluminescence (ECL) system was from Amersham (Uppsala, Sweden). All other reagents were of molecular biology grade.

Study samples and cell lines

A total of 40 subjects were recruited for this study. These included: (1) 20 AD patients (Table 1), with moderate to severe disease. Patients were diagnosed in the department of Neurology of the University Hospital Doce de Octubre (Madrid, Spain) according to the National Institute of

Neurological and Communicative Disorders Association criteria [34]. Cognitive status was quantified using the Mini-Mental State Examination (MMSE); patients were diagnosed of moderate AD (DSM-III-R, MMSE score between 10 and 18) or severe AD (MMSE < 10). They were all considered sporadic, late-onset AD cases (onset of symptoms >65 years; family history negative for neuropsychiatric disorders). All AD patients presented a 1–6 year history of progressive cognitive impairment predominantly affecting memory; (2) 20 healthy subjects, matched for age distribution, without history of degenerative or cerebrovascular diseases, and without cognitive impairment or other neurological disorders. A summary of demographic characteristics of all subjects enrolled in the study is reported in Table 1.

Establishment of lymphoblastoid cell lines was performed in our laboratory as previously described [35], by infecting peripheral blood lymphocytes with the Epstein-Barr virus [36]. Cells were grown in suspension in T flasks in an upright position, in approximately 10 ml of RPMI-1640 (Gibco, BRL) medium that contained 2 mM L-glutamine, 100 μg/ml penicillin/streptomycin and, unless otherwise stated, 10% (v/v) fetal bovine serum (FBS) and maintained in a humidified 5% CO_2 incubator at 37°C. The medium was routinely changed every 2 days.

Cell survival assay

The cell suspension was mixed with a 0.4% (w/v) Trypan Blue solution, and the number of live cells was determined using a hemocytometer. Cells failing to exclude the dye were considered nonviable. In some experiments, cell viability was checked by the MTT assay [37], obtaining similar results.

Assessment of apoptosis and caspase activity

An apoptosis-detection kit that measured phosphatidylserine (PS) was purchased from Pharmingen (San Diego, CA). The assay was conducted following the manufacturer's directions. Cells were analyzed for phosphatidylserine (PS) exposure/propidium iodide (PI) exclusion by staining with FITC-Annexin V and PI. The activation of executive caspases was investigated using the Vybrant FAM Caspase-3 and 7 Kit (Invitrogen) including FLICA reagent that is retained within the cell, if bound to the active caspase molecule. Control and AD lymphoblasts were resuspended in 300 μl of RPMI containing 10 μl of FLICA reagent and incubated in 5% CO_2 at 37°C for 60 min. The cells were then washed with, and suspended in, wash buffer provided with the kit. The samples were analyzed on the flow cytometer.

Immunoblotting analysis

For Western blot analysis, 50–100 μg of protein from whole-cell extracts were fractionated on a SDS polyacrylamide gel, and transferred to PVDF membrane (Immobilon-P). The amount of protein and the integrity of transfer were verified by staining with Ponceau-S solution (Sigma). The membranes were then blocked with 1% BSA and incubated overnight at 4°C with primary antibodies at the following dilutions: 1:500 anti-phosphorylated ERK1/2, 1:2,000 anti-total ERK1/2, 1:500 anti-phosphorylated Akt, 1:1,000 anti-total Akt, 1:500 anti-p21 anti 1:5,000 β-actin. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Sigma) and detected with a chemiluminescent substrate detection system ELC (Amersham). The relative protein levels were determined by scanning the bands with a GS-800 imaging densitometer provided with the Quantity One 4.3.1 software from BioRad.

Statistical analysis

Unless otherwise stated, all data represent mean ± standard error of the mean (SE). Statistical analysis was performed on the Data Desk package (version 4.0) for Macintosh. Statistical significance was estimated with Student's t test or, when appropriated, by analysis of variance (ANOVA) followed by Fischer's LSD test for multiple comparisons. Differences were considered significant at a level of $p < 0.05$.

Results

Effect of SIM on serum deprivation-induced cell death in lymphoblasts from control or AD subjects

Figure 1 shows a time-course analysis of the effect of increasing doses of SIM on rates of cell death, upon serum deprivation, of lymphoblasts from control and AD patients. In agreement with previous reports from this laboratory, it was found that lymphoblasts from AD patients were more resistant to cell death induced by serum deprivation [33, 38]. Here, we show that SIM treatment sensitizes AD lymphoblasts to cell death. The highest concentration tested had little effect in enhancing the death of control cells induced by serum deprivation (Fig. 1, upper left panel). However, in AD lymphoblasts, there is a dose–response effect of SIM inducing cell death in lymphoblasts from AD patients (Fig. 1, upper right panel).

For determining the role of various isoprenoids derived from MEV in regulating the modulatory effect of simvastatin on survival of serum-deprived AD lymphoblasts,

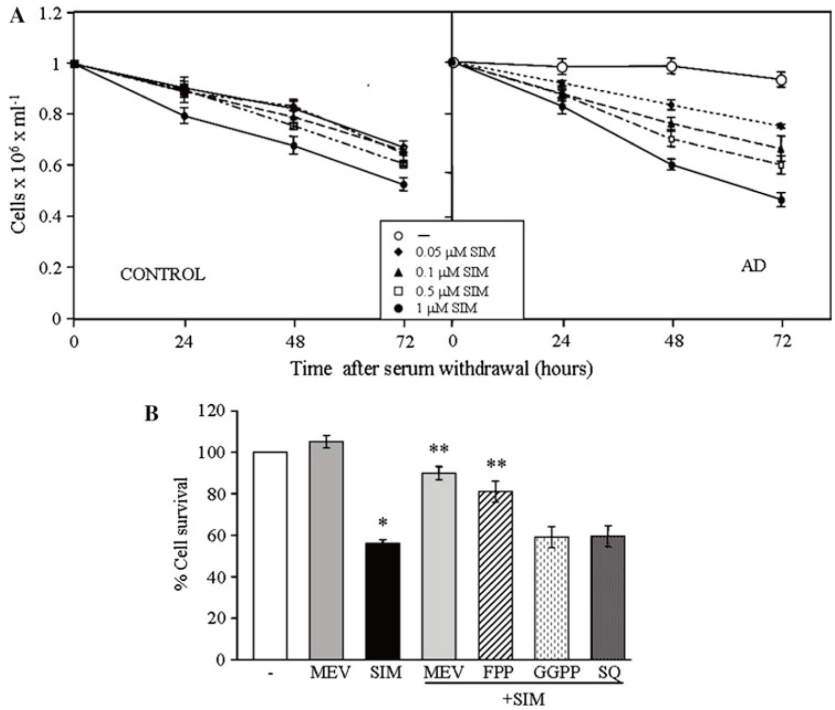

Fig. 1 Effect of SIM on cell survival following serum deprivation in lymphoblasts derived from control or AD patients. **a** Immortalized lymphocytes from control and AD individuals were seeded at an initial density of 1×10^6 ml^{-1} and incubated in serum-free RPMI medium for 72 h in the absence or in the presence of increasing concentrations of SIM. Every day thereafter, cell viability was determined by Trypan Blue exclusion under inverted phase-contrast microscopy. Data shown are the mean ± SE of four to ten independent experiments carried out with cell lines from different individuals. **b** Lymphoblasts from AD subjects were seeded at an initial density of 1×10^6 ml^{-1} and cultured for 3 days in the absence or presence of 1 µM SIM alone or in combination with 200 µM MEV, 5 µM FPP, 5 µM GGPP, or 5 µM SQ. Cell survival was determined by enumeration of cells excluding Trypan Blue. The percent of cell survival is shown after setting the survival of untreated cells as 100%. Values shown are the mean ± SE for four to ten independent experiments carried out with cells derived from different individuals. *$p < 0.01$ significantly different from untreated cells; **$p < 0.01$ significantly different from SIM-treated cells

cells were cotreated with 1 µM SIM and MEV or various isoprenoid intermediaries GGPP, FPP, and SQ. Figure 1b shows that MEV reversed the effect of SIM on cell death. The cotreatment of AD cells with FPP, but not GGPP or squalene (SQ), prevented the SIM-induced apoptosis (Fig. 1b), suggesting that the effect of SIM is independent of cholesterol biosynthesis, and the involvement of farnesylated proteins in the process.

SIM induced apoptosis in AD lymphoblasts

The cell death induced by SIM in serum-deprived AD lymphoblasts showed characteristics of apoptosis. First, a FACS analysis using FITC-AnnexinV/PI double staining showed that the addition of SIM to AD lymphoblasts mainly increased the percentage of cells in early apoptosis (Fig. 2). It is also shown that the effect of SIM is prevented by the pan-caspase inhibitor z-VAD-fmk (Fig. 2). Moreover, fluorescent cell distribution using FLICA green fluorescent probe indicates that SIM increased the activity of executive caspase 3 and 7 (Fig. 3) as FLICA binds irreversibly to these enzymes when are activated, thus increasing the fluorescence signal in apoptotic cells.

Serum deprivation-induced apoptosis is accompanied by changes in p21 protein levels

p21 has been shown to play an important role in regulating apoptosis in a number of cell types [25, 26]. For this reason, we first evaluated whether serum deprivation induces changes in the cellular levels of p21 in control and AD lymphoblasts, and second, we investigated the effect of SIM on p21 content. Figure 4a shows a time-course analysis of serum withdrawal-induced changes in p21 levels. p21 content increased transiently, with a peak level at 24 h of the serum deprivation and returning to basal levels at

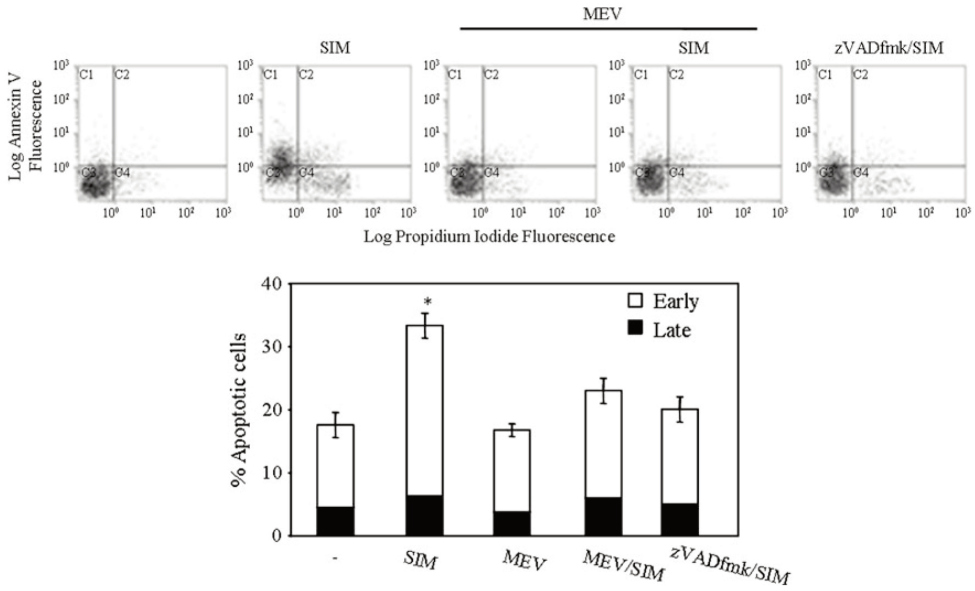

Fig. 2 Flow cytometric analysis of the fraction of viable, apoptotic, and necrotic cells after treatment of AD lymphoblasts with SIM. Lymphoblasts from AD subjects were incubated as described in the legend to Fig. 1, and then stained with FITC-Annexin V and PI. A representative experiment is shown. When present, the concentration of z-VAD-fmk was 1 μM. The means of eight independent experiments carried out with cells derived from different individuals are shown below. *Statistically significant ($p < 0.02$) difference of the proportion of apoptotic cells compared to untreated cultures

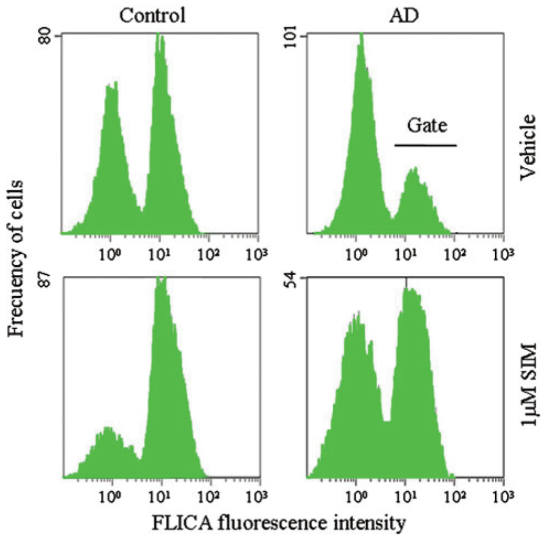

Fig. 3 Caspase activation in serum-deprived lymphoblasts from control and AD subjects. Effect of SIM treatment. Lymphoblasts from control and AD subjects were incubated in serum-free medium for 72 h, in the absence (*upper*) or presence (*lower*) of 1 μM SIM. Then, cells were labeled with the FLICA reagent, following the manufacture's recommendations to detect its binding to active caspases-3 and 7. A representative flow cytometric analysis of the frequency distribution of cells according to their green fluorescence intensity is presented

72 h. The response of control and AD cells was qualitatively identical, but in the latter more p21 accumulated. Thereafter, we focused our subsequent experiments at 48 h of serum deprivation to compare differences in p21 content and cell survival between control and AD lymphoblasts treated with SIM. Figure 4b shows that while SIM slightly decreased p21 levels in control cells, it strongly inhibited the content of p21 in AD lymphoblasts. Addition of MEV to AD cells, in the presence of SIM, partially prevented the effects of the statin, decreasing p21 cellular content. Under these conditions, AD cells treated with SIM undergo significant apoptosis as they do in control cells (Fig. 2). These results suggest that overexpressed p21 may play a role in the resistance of AD lymphoblasts to serum withdrawal-induced cell death, and that SIM is able to overcome this feature by decreasing p21 levels.

Time course of intracellular signaling pathway activation following serum withdrawal

PI3K/Akt and ERK1/2 pathways play a central role in cell death and survival [39, 40]. Both pathways are activated after serum deprivation in the control cells as well as in the AD cells (Fig. 5). Activation was assessed by determining the phosphorylation status of Akt and ERK1/2, by Western-blot analysis. PI3K/Akt activity increased transiently, reaching a peak 24 h after serum withdrawal. Enhanced

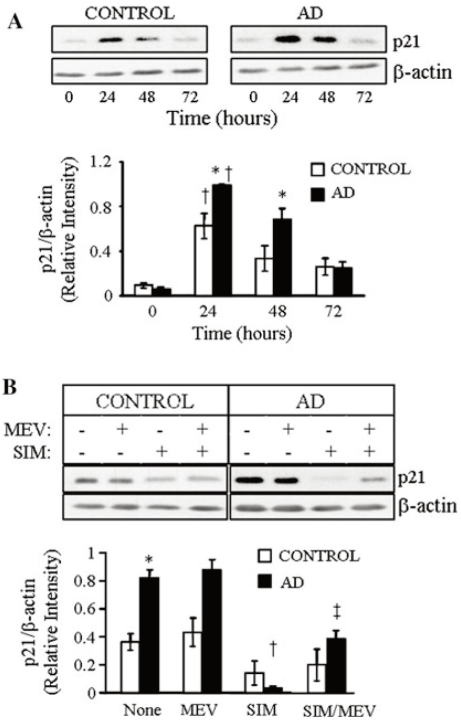

Fig. 4 Serum withdrawal induced a transient increase in p21 levels. Effects of SIM and MEV treatments. **a** Immortalized lymphocytes from control and AD individuals were seeded at an initial density of 1×10^6 ml^{-1} and incubated in serum-free RPMI medium for 72 h. At the times indicated, aliquots were taken to prepare cell extracts. The relative levels of p21 were assessed by Western-blot analysis using anti-p21 antibody. The same membranes were then stripped and reprobed with antibody against *β*-actin. The immunoreactive bands were quantified by densitometric analysis. Results shown below are the mean ± SE of five determinations. **b** Lymphoblasts from control and AD patients were incubated for 48 h in the absence or presence of 1 μM SIM alone or in combination with 200 μM MEV. Results shown are the mean ± SE of six independent experiments carried out in cell lines from different individuals. $^*p < 0.01$ significantly different from control cells; $^\dagger p < 0.01$ significantly different from both control or AD cells before serum deprivation; $^\ddagger p < 0.01$ significantly different from AD cells incubated in the absence of SIM after 72 h of serum deprivation

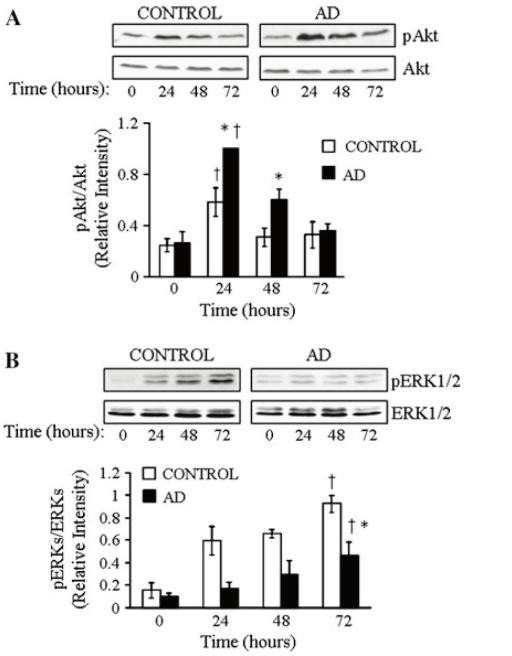

Fig. 5 PI3K/Akt and ERK1/2 signaling pathways are activated by serum deprivation in control and AD lymphoblasts. **a**, **b** Immortalized lymphocytes from control and AD individuals were seeded at an initial density of 1×10^6 ml^{-1} and incubated in serum-free RPMI medium for 72 h. At the times indicated, aliquots were taken to prepare cell extracts. The relative levels of activation of PI3K/Akt and that of ERK1/2 were assessed by Western-blot analysis using phosphospecific antibodies. The same membranes were then stripped and reprobed with antibodies against total Akt or ERK1/2. Representative immunoblots are shown, while densitometric analysis are the mean ± SE of independent experiments carried out in cell lines from different individuals. $^*p < 0.05$ significantly different from control cells harvested at the same time point, $^\dagger p < 0.01$ significantly different from both control or AD cells before serum deprivation

PI3K/Akt activation was found in AD cells compared to control cells (Fig. 5). On the other hand, as previously reported [33], ERK1/2 activity increased progressively in response to serum deprivation in control and AD lymphoblasts, although the phosphorylation of the ERK1/2 was significantly lower in AD cells (Fig. 5).

To evaluate the relationship between alterations in PI3K/Akt and ERK1/2 and apoptosis of control and AD lymphoblasts in serum-free medium in the absence or in the presence of SIM, we use the selective PI3K/Akt and ERK1/2 pathway inhibitors, Ly294002 and PD98059, respectively [41, 42]. Despite the activation of PI3K/Akt following serum deprivation, the treatment of cells with Ly294002 did not change the cell response to serum withdrawal-induced cell death in control cultures (Fig. 6a). In contrast, the inhibitor of the ERK1/2 pathway, PD98059, prevented serum deprivation-induced apoptosis in control cells, which is in agreement with previous work from our laboratory [33]. The effect of SIM-inducing cell death in AD lymphoblasts is not affected by ERK1/2 inhibition (Fig. 6a). However, Ly294002 blunted the effect of SIM-inducing apoptosis in serum-deprived AD cells. Ly294002 slightly increased death of AD lymphoblasts following serum withdrawal (Fig. 6a). We confirmed the effect of these inhibitors on the phosphorylation status of their respective kinases (Fig. 6b). PD98059 decreased ERK1/2 phosphorylation but did not modify Akt phosphorylation, whereas Ly294002 inhibited the Akt phosphorylation

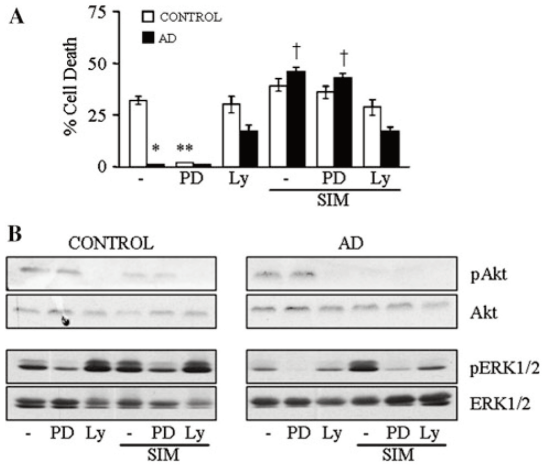

Fig. 6 Effects of SIM on cell viability in the absence and presence of kinase inhibitors. **a** Immortalized lymphocytes from control and AD individuals were seeded at an initial density of 1×10^6 ml^{-1} and incubated in serum-free RPMI medium for 72 h, in the absence or presence of 1 μM SIM with or without 10 μM Ly294002 or 20 μM PD98059. Cell viability was determined by Trypan Blue exclusion. Values shown are the mean ± SE of seven independent experiments carried out in cell lines from different individuals. $^*p < 0.01$ significantly different from control cells; $^{**}p < 0.01$ significantly different from control cells in the absence of PD98059; $^{\dagger}p < 0.01$ significantly different from AD cells in the absence of SIM. **b** The relative levels of activation of ERK1/2 and Akt were assessed by Western-blot analysis using phosphospecific antibodies in cells extract prepared 48 h after serum deprivation. Representative immunoblots are shown

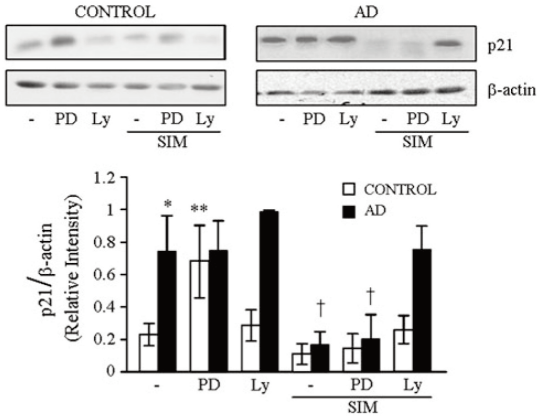

Fig. 7 Effects of SIM on p21 content in the absence and presence of kinase inhibitors. The experimental conditions are identical to those described in the legend to Fig. 6. The cellular levels of p21 were assessed by Western-blot analysis in cell extracts prepared 48 h after serum deprivation. Immunoblots shown are representative of four separate experiments carried out in cell lines from different individuals. Densitometric analyses are shown below. $^*p < 0.05$ significantly different from control cells; $^{**}p < 0.05$ significantly different from control cells incubated in the absence of PD98059; $^{\dagger}p < 0.05$ significantly different from AD cells in the absence of inhibitors

without affecting ERK1/2 phosphorylation status. In addition, we observed that SIM decreased Akt phosphorylation in both control and AD lymphoblasts and it rescued the decreased ERK1/2 activation in AD cells following serum withdrawal (Fig. 6b). SIM did not affect the levels of total ERKs or Akt. The effects of these inhibitors alone or on combination with SIM in the cellular content of p21 are presented in Fig. 7. Inhibition of ERK1/2 activity by PD98059 in control cells caused an increase in p21 levels (Fig. 7). However, the presence of PD98059 did not block the effect of SIM-reducing p21 content (Fig. 7), and impaired the protective effect of PD98059 on the serum withdrawal-induced apoptosis in control cells (Fig. 6a). As mentioned, SIM reduced the levels of p21 in AD cells in the absence or in the presence of PD98059 and sensitized AD lymphoblasts to apoptosis (Fig. 6a). The effect of SIM-reducing p21 levels was prevented by Ly294002 (Fig. 7), in consonance with the blockade of the SIM-induced ERK1/2 activation and cell death in these conditions (Fig. 6).

To gain insight into the role played by the PI3K/Akt and ERK1/2 signaling pathways in regulating the cellular content of p21, we compared the kinetics of changes in the activation of these pathways and that of p21 levels following the treatment of cells with the selective inhibitors Ly294002 and PD98059. The results of these time-course experiments are presented in Fig. 8. Treatment of control or AD cells with Ly294002 rapidly inhibited Akt phosphorylation, and modestly decreased the p21 content (Fig. 8a). However, PD98059 progressively inhibited ERK1/2 phosphorylation in control cells, with simultaneous increase in p21 content (Fig. 8b, left panel). Treatment of AD cells with PD98059 had no consequences in the levels of p21 in these cell cultures (Fig. 8b, right panel). Taken together, these results suggest that cell fate (survival or death) ultimately relies on p21 levels, which appear to be controlled by both the PI3K/Akt and the ERK1/2 signaling pathway in an opposite manner. The ERK1/2 pathway seems to play an indispensable proapoptotic role in the serum deprivation-induced apoptosis, while the PI3K/Akt pathway may act as a survival signal.

Discussion

It was previously reported that EBV-immortalized lymphocytes from AD patients from late-onset AD patients show enhanced proliferative activity [28, 43] and appear to be ill-equipped to survive to serum deprivation [33, 44]. These tumor-like features of lymphoblasts from AD

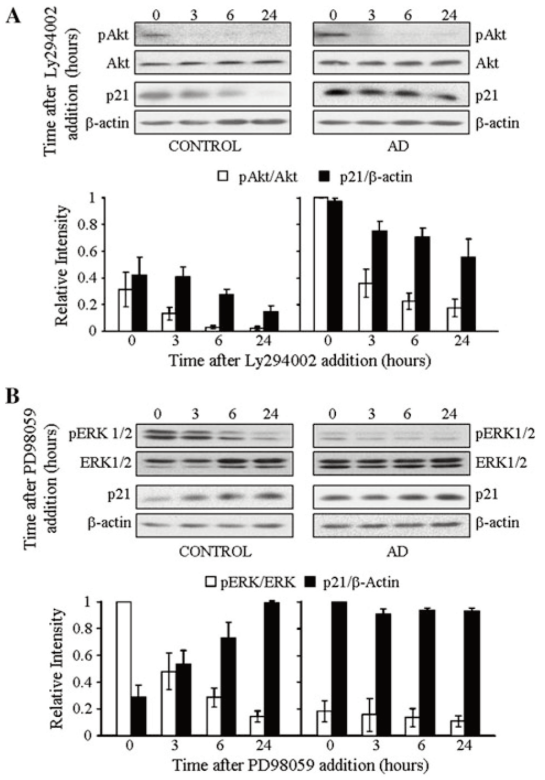

Fig. 8 Comparative kinetics of either PI3K/Akt or ERK1/2 inhibition and changes in cellular p21 levels. **a** Control and AD cells deprived of serum during 24 h, were incubated in the presence of 10 μM Ly294002 for the indicated periods of time. **b** Control and AD cells were incubated in serum-free medium in the presence of 20 μM PD98059 for the indicated periods of time. The immunoblots show the time-dependent effect on Akt phosphorylation or ERK1/2 phosphorylation and p21 content after 3, 6, and 24 h of treatment. The blots are representative of four to eight separate experiments carried out with cell lines form different individuals. Densitometric analyses are shown below

patients were considered as systemic manifestations of the proposed relationship between cellular stress and unscheduled cell-cycle entry observed in susceptible neurons in AD [45]. Moreover, we could demonstrate that the immortalization procedure did not alter the cellular response of fresh obtained lymphocytes to addition or withdrawal of mitogenic factors [33, 43]. Thus, it was considered that EBV-transformed lymphocytes represent a suitable model to study cellular or molecular perturbations of pathophysiological significance.

The present work aimed at studying the influence of statins on the cell fate under serum deprivation of lymphoblasts from late-onset AD patients as compared to that obtained from age-matched non-demented individuals.

The results herein reported confirm and extend our previous work by showing that the survival of AD lymphoblasts is associated with enhanced p21 content upon serum deprivation in comparison to the levels found in control cells. Increased levels of p21 and higher resistance to oxidative stress-induced apoptosis were also found in AD fibroblasts [46]. Although there seems to be a selective impairment of mechanisms involved in cell death in peripheral cells from AD patients [46–49], contradictory results of whether cells from AD patients are more resistant or more vulnerable to situations that promote cell death had been reported. These discrepancies may be due, in part, to the fact that fibroblasts, T or B lymphocytes or EBV-immortalized lymphocytes, from either sporadic or familial AD, exposed to a number of cell-death-inducing conditions have been considered.

A number of recent studies pointed out that in addition to being an inhibitor of cell proliferation, p21 may protect cells from apoptosis [50]. For example, it has been reported that up-regulation of p21 blocked the oxidative stress-induced death of human myeloma U266 cells [51] and that inducible expression of exogenous p21 render glioblastomas resistant to chemotherapy drugs [52]. Thus the increase in p21 cellular content in AD lymphoblasts may confer these cells a survival advantage to these cells as described for cancer cells [53].

SIM treatment slightly increases the serum deprivation-mediated death of control lymphoblasts while sensitizing AD cells to serum withdrawal-induced cell death. In contrast with the effect of SIM increasing the levels of CDK inhibitors, p21 and p27 in AD lymphoblasts under proliferative conditions [32], SIM drastically decreased the p21 content of serum-deprived lymphoblasts from AD. Cell death induced by SIM showed characteristics of apoptosis, since it was prevented by a pan-caspase inhibitor, and showed dependence on caspase-3 and 7 activation.

The higher sensitivity of AD cells to SIM, compared to that of control lymphoblasts, is in line with reports indicating that B leukemic and myeloma cells undergo apoptosis with statins treatment, whereas their normal counterparts are resistant to statin effects [54]. Moreover, statins inhibit the growth of variant human embryonic stem cells and cancer cells in vitro but not the growth of normal human embryonic stem cells [55]. The results described herein are, however, in contrast with the reported neuroprotective effects of statins in neuronal cells [56–58]. These effects of statins, protecting neuron from certain noxious stimuli, involve alteration in the ratio of pro and anti apoptotic proteins and inactivation of caspases [59]. Although lower concentrations of statins (<1 μM) were used in treating cortical neurons [58], the little effect of concentrations of SIM (up to 1 μM) in control lymphoblasts rules out a cytotoxic effect of the drug. Taken

together, it seems that the effects of SIM, inducing or preventing apoptosis, are cell type-specific, and perhaps dependent on the nature of the stimulus.

Despite the pro-apoptotic effect of SIM on AD lymphoblasts, a neuroprotective effect of SIM in AD brain cannot be fully discarded. In this regard, it is worth mentioning that SIM addition to AD cells was able to restore the "normal" cell response to serum stimulation [32] or withdrawal (this manuscript), by blunting the enhanced proliferative activity of AD cells or sensitizing cells to apoptosis in the absence of serum. In both situations, SIM was able to increase [32] or decrease the levels of p21 of AD lymphoblasts to reach those of control cells. It remains to be demonstrated whether SIM would protect neurons in AD brain from apoptosis by modulating p21 content.

The proapoptotic effect of SIM in AD cells is directly related to HMG-CoA reductase inhibition because the effects of SIM on cell viability and p21 content were completely or partially reversed by MEV. It is well known that MEV acts as a precursor to lipid moieties covalently attached to isoprenylated proteins, such as small GTP-ases. By preventing isoprenylation of certain proteins, SIM may regulate essential signaling pathways such as PI3K/Akt or MAPK, which are involved in cell proliferation and survival [2]. The modulatory action of SIM on the serum withdrawal-induced apoptosis reflects interference with farnesylation rather than with geranygeranylation of proteins.

Our results show that serum deprivation induces a transient increase in PI3K/Akt activity together with a sustained activation of the ERK1/2 pathway in both control and AD lymphoblasts. However, important quantitative differences were observed. While PI3K/Akt activation was enhanced in AD cells, the activity of ERK1/2 was downregulated with the net result of increased p21 levels and higher resistance to cell death. SIM treatment inhibited PI3K/Akt activation in control and AD cells, whereas in the latter, it reversed the activity of the ERK1/2 pathway to levels similar to those found in control cells. This shift away from the PI3K/Akt signaling pathway towards sustained ERK1/2 activation seems to favor cell death over survival. Indeed the ERK inhibitor PD98059 is able to overcome the serum deprivation-induced apoptosis in control cells, however inhibition of PI3K/Akt by Ly294002 had only a minor effect on the survival of AD cells. The SIM-induced apoptosis of AD cells was blocked in the presence of Ly294002 and, on the other hand, SIM prevented the effect of PD98059 protecting control cells from apoptosis induced by serum deprivation. These results suggest that both pathways cooperate to cell decision. Cell survival was always associated with decreased ERK1/2 phosphorylation levels, together with little change or even inhibition of PI3K/Akt provided that p21 content was maintained over certain levels. The proposed scenario is represented schematically in Fig. 9. The interaction of SIM with the ERK1/2 and PI3K/Akt signaling pathways results in a significant reduction of p21 content and apoptosis of AD lymphoblasts following serum withdrawal. In control cells, the balance between ERK1/2 and PI3K/Akt following serum deprivation is tipped in favor of ERK1/2 activation-induced p21 decreased levels and apoptosis. In these conditions, the inhibition of PI3K/Akt by SIM had only a moderate increase in the cell death induced by serum withdrawal.

Overexpressed p21 could protect cells from apoptosis at different levels. First, p21 is shown to induce the expression of antiapoptotic proteins [26]. Second, p21 interacts with several caspases [25, 60]. Third, it is possible that p21 can protect against oxidative stress-induced toxicity and thus promote survival of cells [61]. Which of these mechanisms predominates in lymphocytes is not yet known and needs further experimentation.

It is worth mentioning that overexpressed p21 has also been detected in the frontal cortex of AD brains [62]. It is known that monocytes can protect themselves from oxidative stress by upregulating the cytosolic levels of p21 [63]. It is therefore tempting to speculate that neurons in AD brain facing conditions of oxidative stress could initiate a similar compensatory response to protect the injured cells from death.

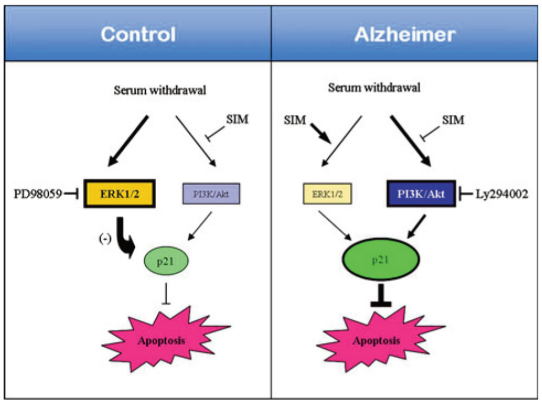

Fig. 9 Diagram summarizing the interaction of SIM with ERK1/2 and PI3K/Akt pathways in control and AD lymphoblasts under conditions of serum deprivation. **a** In control cells, serum withdrawal promotes apoptosis by inducing sustained overactivation of ERK1/2 which in turn reduces p21 accumulation. **b** In AD cells, in the absence of SIM, the ERK1/2 pathway is downregulated, and the activity of PI3K/Akt is enhanced relative to control cells, as well as it is the p21 cellular content. Treatment of AD cells with SIM prevented serum deprivation-dependent overactivation of PI3K/Akt, increased ERK1/2 activity, normalized p21 levels, and sensitized AD cells to the serum-induced apoptosis

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Finally, alterations in PI3K/Akt and ERK1/2 signaling pathways and changes in the abundance of p21 similar to those described in immortalized lymphocytes from AD patients, has been detected in brain from affected individuals [62, 64–66], thus suggesting that peripheral cells from patients may be a potential useful surrogate for diagnosis, prognosis, and therapeutic monitoring of AD.

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